The quantitative estimation of metals based on chromatographic separation

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The Quantitative Estimation of Metals based on Chromatographic Separation.

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PREFACE

Since the appearance in 1944 of the now classic article on paper chromatography by A.J.P. Martin, there has been a remarkable growth both in qualitative and quantitative chromatography. The object of this present work is to give a brief account of the principal chromatographic methods which have been used in estimating metals, and in particular to study certain fundamental aspects of photometric estimation carried out directly on paper.

Chromatography is an elegant and speedy method of analysing many mixtures, which had previously proved intractable. In the quantitative field, photometric methods of estimating substances directly on paper are rapid, general in application, and capable of considerable accuracy. They require, however, close attention to a large number of factors, and this has restricted their widespread adoption. The writer's experimental work is aimed at studying some of these factors, and an attempt has been made to draw comparisons between different photometric methods.

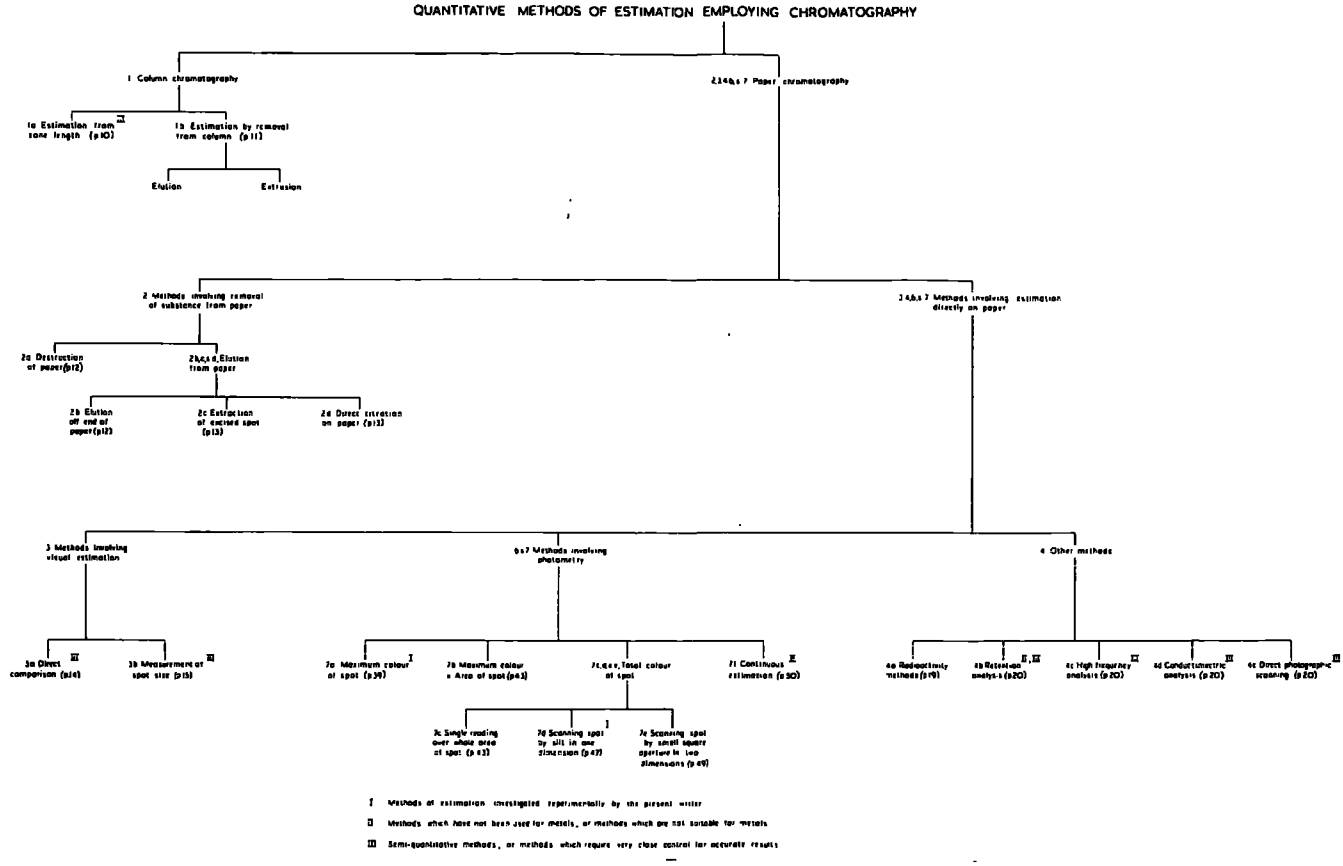
CONTENTS

A. INTRODUCTION

Chromatography was first investigated in the 19th Century by F. F. RUNGE[1] (1850) and C. F. SCHOENBEIN[2] (1861) who separated dyes and inorganic substances on paper. The Russian botanist M. TSWETT[3] (1906), who separated pigments on adsorbent columns, is however generally regarded as the originator of chromatography in its modern form. Column chromatography using adsorbents was greatly developed during the next forty years, but paper chromatography was not used in this period. In 1941 A. J. P. MARTIN[4] employed silica gel which acted as a stable support for a water phase, instead of an adsorbent such as alumina, thus making possible chromatography employing two liquid phases. In 1944, an attempt by Martin[5] to find a more suitable stable support led to the introduction, or re-introduction, of paper chromatography. The advantages of paper chromatography were quickly realised and have led to the development of numerous methods of quantitative estimation.

B. LITERATURE SURVEY

QUANTITATIVE METHODS OF ESTIMATION EMPLOYING CHROMATOGRAPHY



I Methods of estimation investigated experimentally by the present writer

II Methods which have not been used for metals, or methods which are not suitable for metals

III Semi-quantitative methods, or methods which require very close control for accurate results

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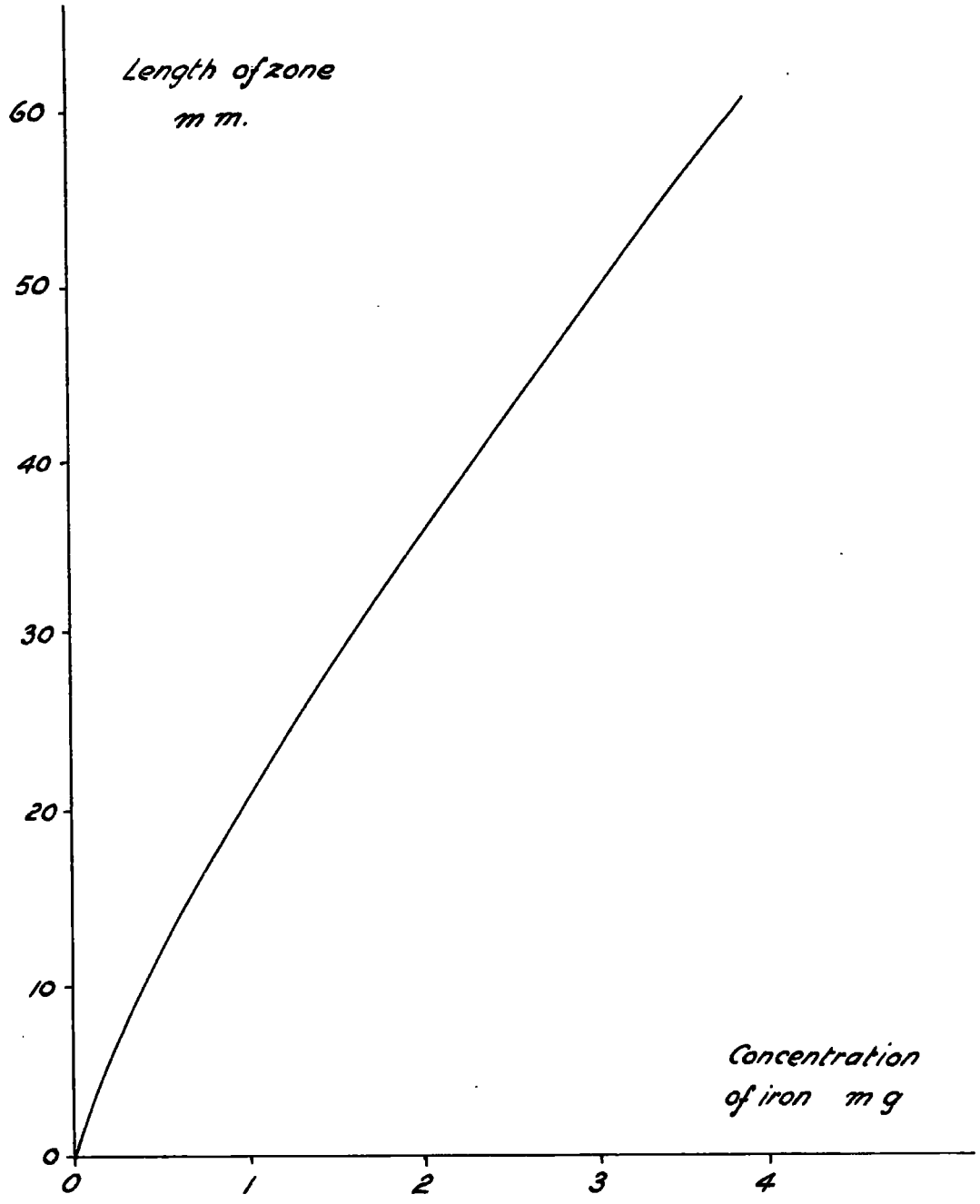

Fig. 1. Relation between length of zone and concentration of iron (Z. Pinterovic[8])

§ 1

COLUMN CHROMATOGRAPHY

Column chromatograms may be estimated either by measuring zone length or by removal of the substance from the column

(a) Estimation from zone length

As early as 1938, G. M. SCHWAB[6,7], suggested that it was possible to estimate certain metals (e.g. copper and cobalt) by measuring the length of the zone. The method, although simple in principle, required careful standardisation of conditions. Z. PINTEROVIC[8] (1941) used this method in estimating iron on alumina columns. A curve of zone length versus concentration is shown in Fig.1. Other workers who have used this method include G. ROBINSON[9,10] (nickel and zinc), B. S. SRIKANTAN[11,12] (copper), N. TIKOMIROFF[13] (niobium and tantalum), and V. D. KOPYLOVA[14] (ferric iron).

The advantages and disadvantages of this method of estimation are:-

Advantages

(i) The method is simple and no special apparatus is needed.

(ii) Long periods of equilibration are not required, as is sometimes the case with paper chromatography.

Disadvantages

(i) The relation between the zone length and the concentration is not necessarily linear.

(ii) The lengths of the zones are, in many cases, subject to small changes in pH, and to the presence of other ions.

(iii) The method is limited in application because it is necessary to produce really sharp zones.

(b) Estimation by removal from column

The substance may be removed, either by elution through the column, or by excision of the zone followed by extraction of the substance. Both these methods are more widely applicable than the method of estimation based on zone length, and do not usually require such careful standardisation of conditions. The estimation may be carried out by titration, colorimetry, spectrophotometry, or indeed any standard method of analysis. The elution method has been used by G. ROBINSON[9] for copper, by N. TIKOMIROFF[13] for tantalum, and by H. BALLCZO[15] for barium. The excision method has been used by J. E. DUNABIN[16] in estimating traces of cadmium.

§ 2

ESTIMATION BY REMOVAL FROM PAPER

A method of estimating paper chromatograms, which has been widely studied, involves the removal of the substance from the paper before estimation. This may be done in four ways; by destruction of the paper, by elution of the substance from the end of the paper, by extraction of the excised spot, or by the so-called 'direct titration' method. In all cases the actual estimation is carried out in solution by a standard method such as colorimetry or polarimetry.

(a) Destruction of paper

J. A. LEWIS[17] (1951) estimated numerous metals by cutting out the sections of paper containing the developed spots, and ashing them in separate crucibles. The ash was then dissolved in an electrolyte, and the solution was analysed polarographically.

(b) Elution from end of paper

The principle of this method is to elute the substance off the end of the paper into a container. This was carried out by M. LEDERER[18] in the estimation of thallium, and by N. F. KEMBER[19] in the estimation of gold in the presence of large quantities of platinum metals. The chief limitation here, is that the metal to be estimated must have an R_f considerably greater than that of all other substances in the mixture.

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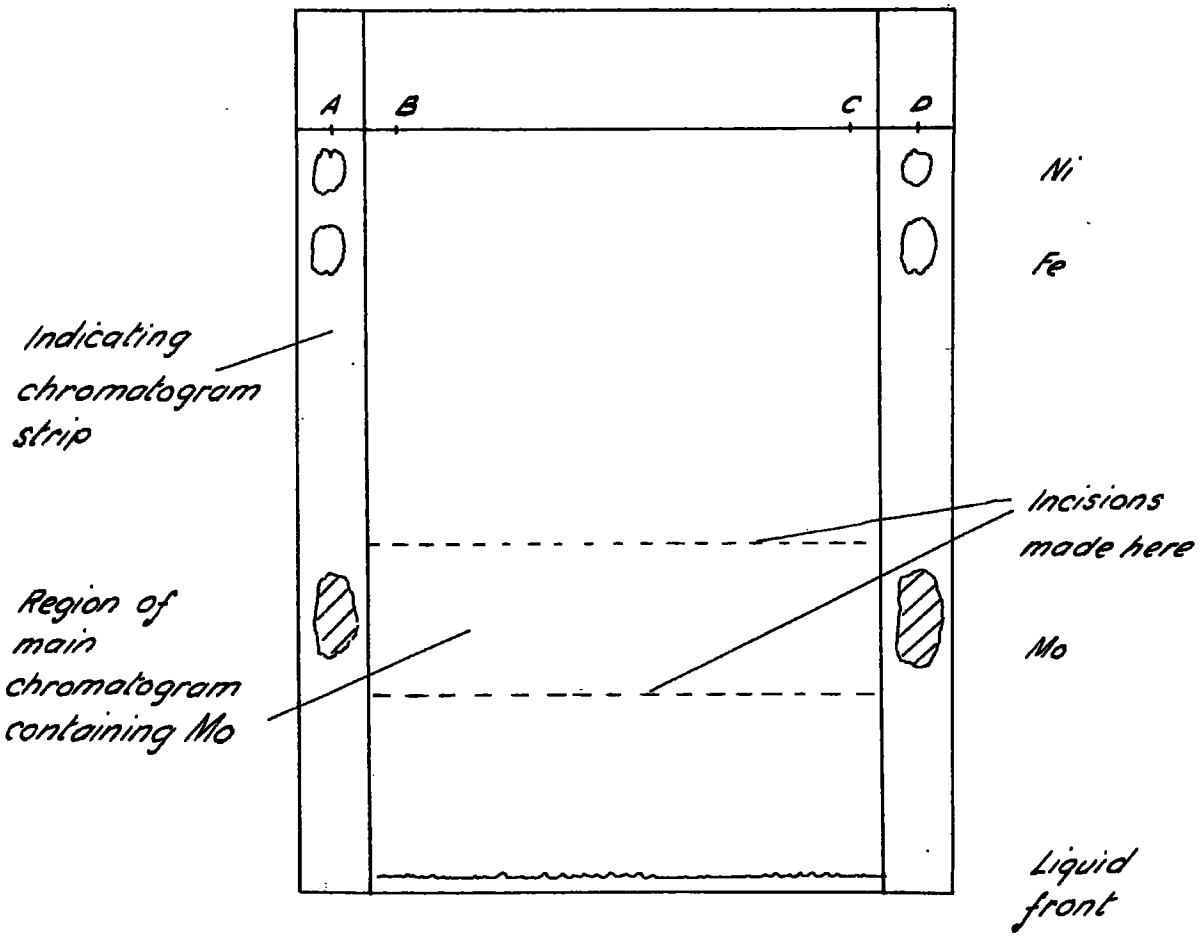

Fig. 2. Diagram showing chromatogram as used by F.H. Pollard.[24]

(c) Extraction of excised spot

This is, perhaps, the most widely practised of all quantitative methods based on paper chromatography. A. LACOURT[20-23] used this method in estimating aluminium, iron, and titanium on thin strips of paper. Under standard conditions, the position of the spot was known, so that it was possible to cut out the sections of the paper containing the various metals, without developing the chromatogram. F. H. POLLARD[24] used a similar method but on broad sheets, using a pilot strip to indicate the position of the spot, as shown in Fig.2. The main part of the chromatogram was then cut out and the metal extracted and estimated. Modifications of this method have been used by I. I. M. ELBEIH[25] in estimating thorium and uranium, and F. H. POLLARD[26] in estimating barium and strontium.

This is a general method capable of giving accurate results. The chief disadvantages are the time taken for the removal of the metal from the paper and the possibility of incomplete removal. These disadvantages do not apply to estimations made directly on paper.

(d) Direct titration

A. LACOURT has applied this method to the estimation of vanadium[27] (1953), molybdenum[28], and zinc[29]. The section of the paper containing the metal to be estimated was cut out of the chromatogram and was placed in a cell containing a solution. The solution was then titrated at once against a reagent placed in a micro-burette. The method reduces considerably the time for analysis, but has not, as yet, been much used.

§ 3

VISUAL ESTIMATION

There are two methods by which developed chromatograms may be estimated without the aid of photometric or other instruments. The spots can be estimated either by direct comparison with standards, or by measurement of their size.

(a) By direct comparison

In principle, the method here is to chromatograph the unknown together with standards of different dilutions, all of which are placed on the same sheet of paper. This procedure ensures that both the unknown and the standards are eluted and developed under identical conditions. The density of the spot formed from the unknown is then matched with one of the spots formed from the diluted standard. This method was used by both A. POLSON[30] (1948) and H. K. BERRY[31] (1949) in the estimation of amino acids, and by T. V. ARDEN[32] (1949) in the estimation of uranium.

An alternative method of direct comparison was employed by J. L. AUCLAIR[33] (1952), in estimating amino acids. If a series of standards of a given amino acid in order of decreasing concentration is prepared, and chromatographed under standard conditions, a series of spots of gradually diminishing clarity will be obtained. The concentration of the last discernible spot is noted. In order to carry out the actual estimation, the unknown solution is diluted to form a series of solutions of gradually decreasing concentration. One spot of each solution is placed on each sheet

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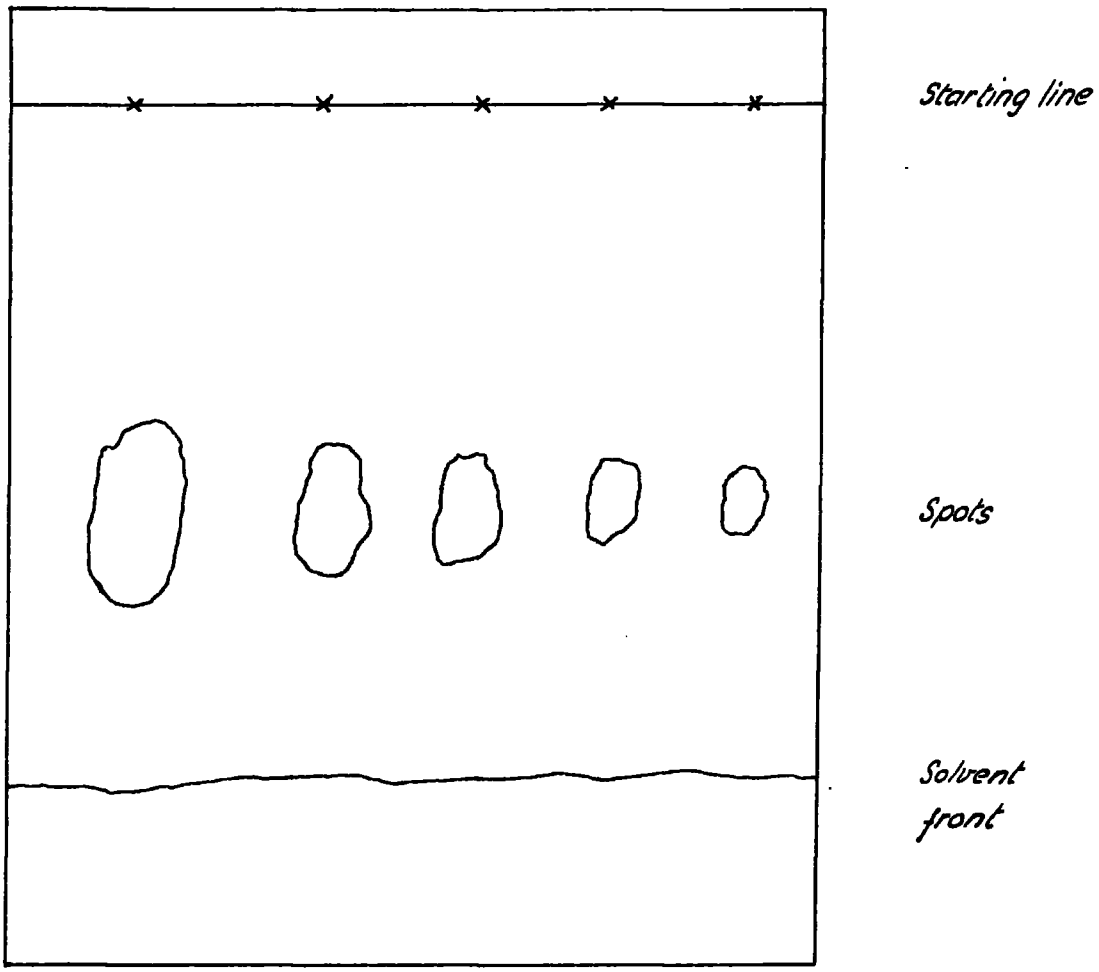

Fig. 3. Appearance of chromatogram containing a series of spots of gradually decreasing concentration

of paper, and all the sheets are eluted and developed simultaneously under standard conditions. The sheets are then inspected by transmitted light, and the dilution of the last discernible spot (for each amino acid) is noted. The concentration of this spot is thus ascertained, and as the dilution of the spot is known, the concentration of the unknown solution can be calculated. This method has the disadvantage of requiring estimation with the minimum quantity of material discernible, which may well be affected to a large extent by the colour due to the developer.

It should be noted that direct comparison methods of estimation suffer from errors due to subjective measurement and need considerable replication. They require, however, no special apparatus and are useful for routine analysis. In general, accuracies of 10 - 30% can be obtained.

(b) Measurement of spot area

If a number of spots of decreasing concentration are placed on a sheet of paper, eluted, and developed, it will be found that they form a series, as shown in Fig.3. The most concentrated solutions produce the largest spots, while the least concentrated produce the smallest spots. The size of the spot (either length or area) may be made the basis of a method of estimation.

The relation between the area and concentration of the spot was first treated theoretically by R. C. BRIMLEY[34](1949). Although the fundamental assumptions have been questioned, the treatment gives valuable insight into the method. Brimley supposed that the spot spreads only by diffusion as it moves along the chromatogram in a way analogous to that in which heat flows along a conductor. Since the original spot is of finite size, the flow by diffusion may be compared to the flow of heat from a finite heat source.

(The diffusion process is comparable to the flow of heat - as both are subject to Fick's law). For a spot of initial concentration c_i of radius r, we have

$$c = \frac{c_i}{2Dt} \cdot \exp\left(\frac{-r^2}{4Dt}\right) \cdot \int_0^a \exp\left(\frac{-r'^2}{4Dt}\right) \times I_0\left(\frac{rr'}{2Dt}\right) r' \cdot dr',$$

where c is the concentration at radius r at time t and D is a diffusion constant. Putting F for the integral containing the modified Bessel function and remembering that $r^2/4Dt$ equals $A/4\pi Dt$, where A is the area of the spot, and taking c as the concentration at the limit of visibility, that is at the edge of the spot, we can take logarithms (to base 10) and rearrange to become

$$A = \frac{4\pi Dt}{\log e}\left[\log c_i - \log c - \log(2Dt) + \log F\right].$$

c, D and t are constant for the same substance in the same chromatogram so this reduces to

$$A = \text{const.}\left[\log c_i + \log F\right].$$

A further simplification can be made which avoids the term log F - a term which increases as A increases - to enable a direct plot of A against log c_i to be made. Brimley calculated the error in the determination of c_i from a linear plot of A against log c_i for various values of a and $2Dt$ and showed the importance of keeping $2Dt$ large and a small. We should thus expect that using a small initial

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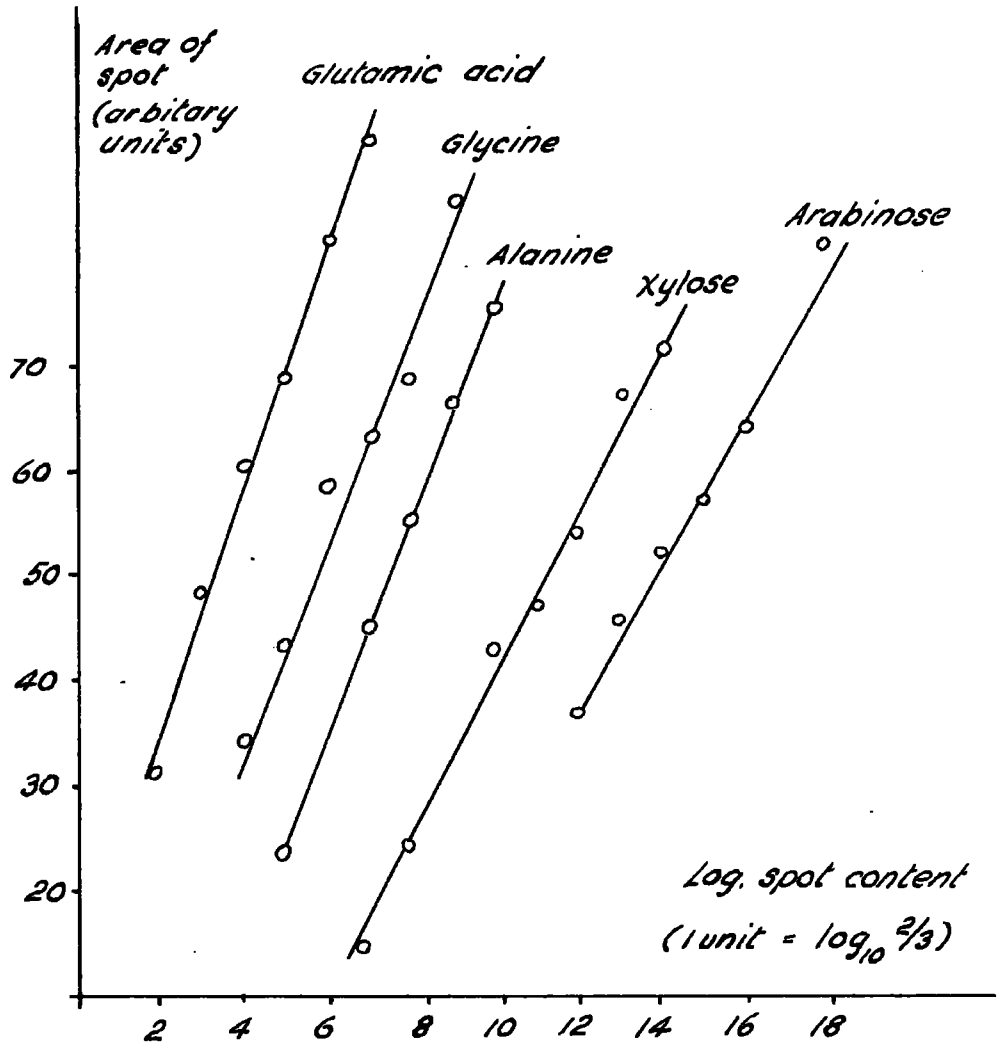

Fig. 4. Estimation of amino acids from area of chromatographed spots (R.B. Fisher [37])

spot and a reasonably long run that a straight line plot would be obtained of A against log c_i over fairly wide limits.

This treatment was criticised by R. B. FISHER[35], who pointed out that diffusion is not the only agency causing distribution of the spot during elution. Also, according to the above treatment, circular spots should be obtained, whereas in practice elliptical spots are invariably produced. J. C. GIDDINGS[36] (1959) has put forward a more complete theory, which accounts for the elliptical shape of the spots.

In 1948, R. B. FISHER[37] had noted that there was a linear relation between the spot area and the logarithm of the spot concentration, in estimating amino acids. 5μl volumes of standards and unknowns were placed side by side on the same sheet of paper, which was then eluted, developed, and dried. The areas of the spots were measured by planimetry and the standards were used to plot a calibration curve from which the concentrations of the unknowns were found by interpolation (Fig.4). An important limitation of the method is that the spots must be sharp and uniform, if accurate results are to be obtained.

R. B. FISHER[37] also noted that there was a linear relation between the concentration and the length of elliptical spots.

Several workers have applied the method of Fisher to organic estimations. R. J. BLOCK[38] (1950) stated that accurate results could be obtained by this method provided that the spots were clearly separated and sharp. On the other hand it was found that more accurate results could be obtained with diffuse spots by the maximum density method. Other workers who have applied area methods of estimation

include R. L. REID[39] (1951) estimating fatty acids, E. BEERSTECHER[40] (1950) estimating trace potassium in urine, and H. SEILER[41] (1952) estimating alkali and alkaline earth metals, particularly in mineral waters.

B. M. TURNER[42] (1958) applied the area method of estimation on the sub-microgram scale to the estimation of zinc, lanthanides, iron, copper, lead, and barium on freshly blown glass surfaces. A liquid film incorporated both the eluting and developing agents. The chromatograms, which were rapidly formed, were measured by two-beam reflection interferometry.

Radial chromatography (a technique described by L. RUTTER[43] (1948)) was used by G. VENTURELLO[44] (1952) who investigated the relation between band width and concentration. Under certain conditions, these factors were found to be linearly related. The method was used in estimating elements in aluminium alloys[45]. E. BOVALINI[46] (1959) used a similar method in estimating Al, Ti, Mn, Co, Cu, and Zn in iron oxide, but made use of a linear relation between band width and the logarithm of the concentration.

§ 4

NON-PHOTOMETRIC METHODS

This section is devoted to certain instrumental methods of estimation which are carried out directly on paper and which do not employ photometry. Of these, radioactivity methods are the most important, although they are at present somewhat limited in application. The other methods mentioned in this section have not been extensively studied.

(a) Radioactivity methods

Metals which are naturally radioactive or contain radioactive tracers are readily detected by passing the chromatogram over a Geiger counter. Radioactivity scanning adaptors have been described by D. R. BANGHAM[47] (1956), D. V. COHN[48] (1955), and J. C. BOURSNELL[49] (1950). If the output is applied to a pen recorder under which a sheet of paper is drawn, a counting rate-curve is obtained which may be used to evaluate the chromatogram quantitatively. The output should be proportional to the quantity of material, and thus the difficulties due to the non-linear relation between concentration and absorption of light inherent in optical scanning are not experienced.

Non-radioactive substances may be analysed by radioactivity methods if a radioactive developer is used. P. C. van ERKELENS[50] (1953) estimated trace metals on paper chromatograms by development with radioactive hydrogen sulphide.

(b) Retention analysis

This method of analysis was applied to organic substances by T. WIELAND[51,52] (1948). If an eluted chromatogram is dipped into another solvent containing substances which react with the substances on the paper, the rate of flow of the second solvent is retarded. This results in gaps in the solvent front, the size of which may be used to estimate the concentration of the substance in the original chromatogram.

(c) High frequency analysis

Y. HASHIMOTO[53] (1952) and I. MORI[54] (1953) detected metals on paper chromatograms by passing the paper between the plates of a tuned-grid high frequency circuit. The presence of metals was indicated by a change in grid current. It would appear that this method has possibilities in quantitative estimation.

(d) Conductimetric analysis

G. de VRIES[55] (1954) used this method to detect alkali metals on paper chromatograms. After drying, the paper strip was passed between two small steel rollers at a constant speed. A d.c. voltage of 4 to 80 volts was applied between the rollers. The current was measured at three second intervals. The spots revealed themselves as regions of high conductivity. This method would also appear to have quantitative possibilities.

(e) Direct polarographic scanning

A. LANGER[56] (1956) found that by placing a chromatogram on a porous porcelain tube partly immersed in the saturated solution of potassium chloride of a calomel electrode, and using an amalgamated gold wire as the cathode, polarographic

evaluations could be made directly on the paper. An apparatus was described which enabled an eluted chromatogram to be scanned polarographically. The moist paper chromatogram was passed between the tube and the amalgamated gold wire across which a potential of 1·6 to 1·8 volts was applied. A flow of current indicated the presence of reducible substances. The current was found to be roughly proportional to the concentration. This method was applied in the estimation of Co, Cd, Tl, and Pb.

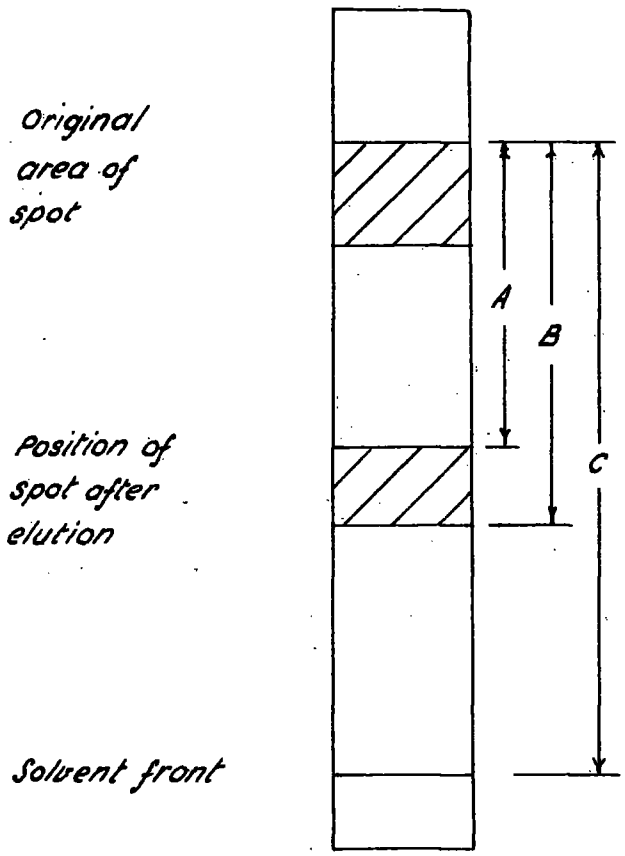

Fig. 5. Measurement of chromatograms used by A. Lacourt[21]

§ 5

REPRODUCIBLE CHROMATOGRAPHY

All methods of quantitative estimation directly on paper require reproducible chromatograms for accurate analysis, since the results may be markedly affected by slight differences in the developed chromatograms. This is one of the great difficulties of estimation directly on paper, for there are a large number of factors that may affect the running of a chromatogram. An attempt must be made to standardise the conditions as far as is practicable, but it is not usually possible to control them all rigidly. The important thing in practice, is to know which factors markedly affect the running and to standardise these as closely as possible. The importance of controlling the factors which affect the size and shape of the spot, varies according to the method of estimation which is to be used. If the method is one which measures the total quantity of substance in the spot, such as a scanning method, the vigorous control of the shape and size of the spot is less important than in a method involving measurement of the area of the spot, or the maximum density.

Constancy of R_f is generally taken as a criterion of reproducible chromatography. Yet the R_f alone does not give enough information for quantitative work, as it gives no indication of the dimensions of the eluted spot. The method of measuring chromatograms used by A. LACOURT[21] (Fig.5),

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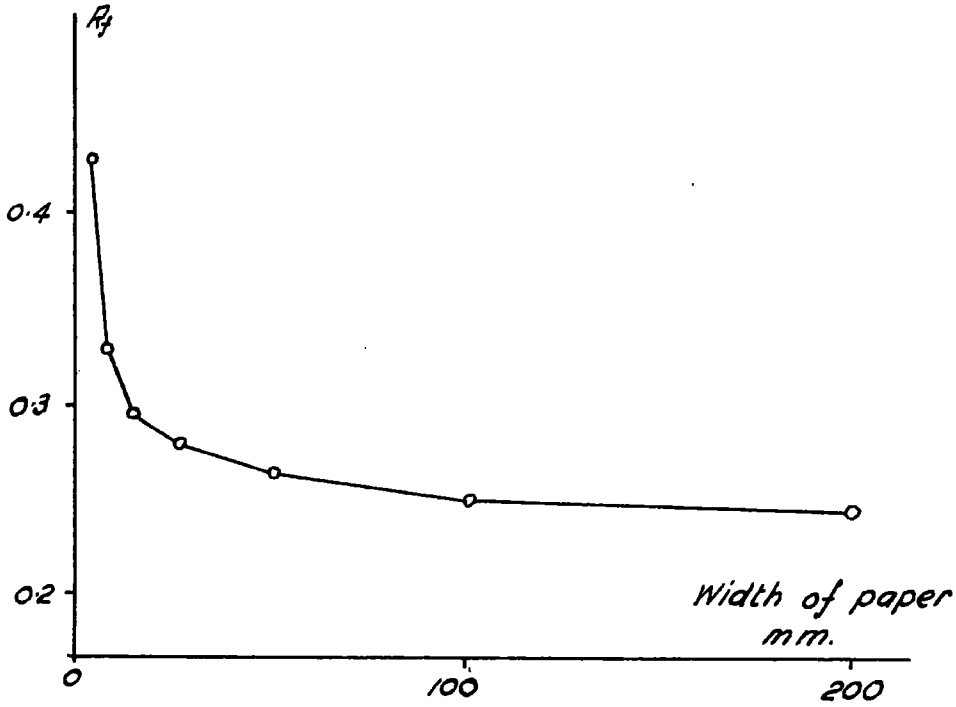

Fig. 6. Effect of width of paper on Rf value of alanine (C.R. Willms[66])

in which the distances A, B, and C are measured is preferable, especially for chromatograms run on thin strips.

Some of the factors affecting the production of chromatograms are now considered.

(a) The nature of the original solution

It is desirable to start with a more or less uniform spot of material on the paper. S. V. VAECK[57] has shown that in the case of nickel, a solution of 3 N hydrochloric acid gives more uniform initial spots than neutral solutions.

The R_f, and also the size of chromatographed spots may be markedly affected by the nature of the original solution. Several workers[20,40,58,59,60] have investigated the effect of varying the acid content and the accompanying anion of the original solution. In most cases, it is best to make up the substance in acid solution, as this usually produces sharper chromatographed spots.

(b) Nature of paper used

Filter paper is subject to considerable variation. A most important property of paper, from the point of view of reproducible chromatography, is uniformity or homogeneity. This is regulated during manufacture by controlling the degree and nature of the beating. Generally the longer the beating time, the shorter are the resulting fibres, although very short fibres may also be obtained by beating the paper for a short time with very sharp beater bars. Highly beaten papers with short fibres are more uniform in texture, but their rate of flow is slower.

Most workers[59,61,62] have found that the slower papers give rather sharper spots. For certain separations acid washed papers are desirable[26,63].

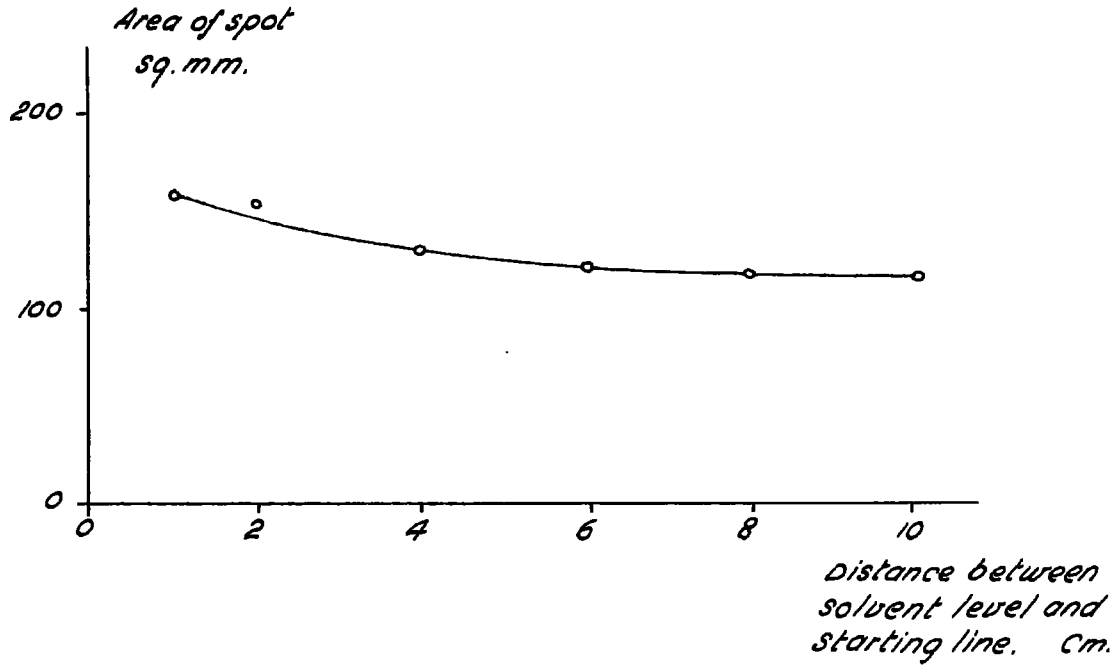

Fig. 7. Effect of starting point position on area of chromatographed spot (A. Lewandowski [68])

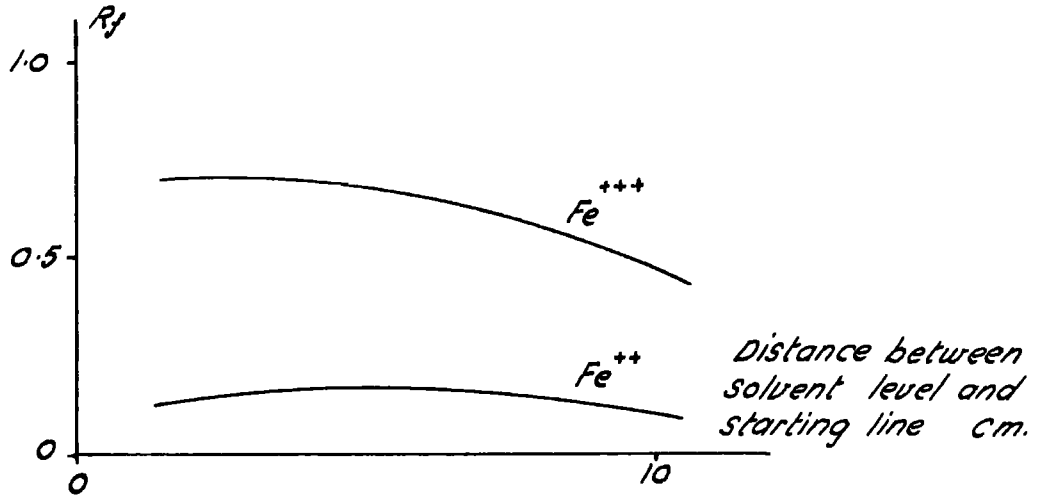

Fig. 8. Effect of starting point position on Rf of Fe^{++} and Fe^{+++} (F. H. Pollard [63])

The rate of flow of papers is usually greater in the machine direction of the paper, but some workers[64] have found that more uniform spots are obtained by allowing the solvent to flow at right angles to the machine direction.

(c) Time of drying spot after deposition

The running of a chromatogram may be affected by a variation in the time for which the spots are dried before placing the paper in the chromatography tank[23, 65].

(d) Shape of paper

The most common shapes of paper used are wide rectangular sheets (which enable several spots to be run on the same sheet of paper) and narrow strips. The R_f values obtained on wide sheets are not necessarily the same as those obtained on narrow strips. Lacourt favoured narrow strip chromatography, although most workers in the quantitative field have preferred wide sheets. The R_f value varies considerably with the width of the paper on narrow strips. C. R. WILLMS[66] has shown that for amino acids separated on narrow strips, the R_f rises rapidly with decreasing width (Fig.6).

(e) Initial position of the spot on the paper

Spots which are placed close to the edge of a broad sheet of paper may run faster than those in the centre. This has sometimes caused difficulty when indicator strips have been used[26,67].

The effect of varying the distance of the spot from the initial level of the solvent has been investigated by A. LEWANDOWSKI[68] and by F. H. POLLARD[63]. Examples of the way in which the area and the R_f may be affected are shown in Figs.7 and 8 respectively.

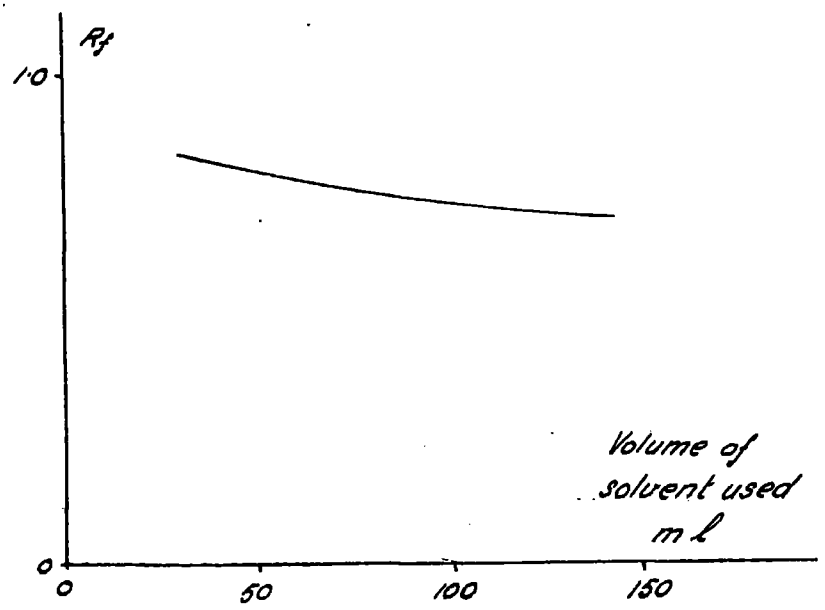

Fig. 9. Effect of volume of solvent used to saturate gas jar in ascending chromatography on R_f value (F.H. Pollard [63])

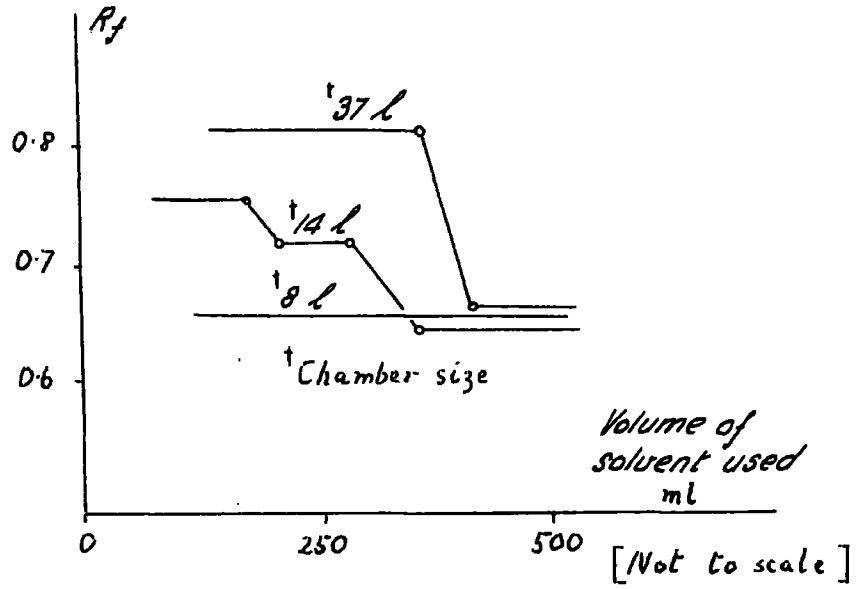

Fig. 10. Effect of volume of solvent used to saturate large tanks on R_f values of amino acids (R.A. Clayton [70])

(f) Degree of saturation of the tank and time of equilibration

A saturated atmosphere is usually regarded as essential for reproducible chromatography. Long periods of time are sometimes required to ensure complete saturation of the air space. In order to speed up evaporation, sheets of filter paper wet with the solvent, may be hung in the tank or alternatively the air in the tank may be fanned[69].

Both the quantity of the solvent used for saturation and the size of the tank used may affect the R_f values. This is probably connected with the degree of saturation of the air space (F. H. POLLARD[63], R. H. CLAYTON[70]). Examples of the effect of these factors on R_f values are shown in Figs. 9 and 10.

In some cases, the effect of allowing the paper to stand in the solvent vapour has such a marked effect on the elution as to alter the sequence of R_f values[71]. Many workers place the spotted sheets of paper in the tank and close the tank, adding the eluting solvent after a few hours through a hole in the lid. This is a much sounder method as it avoids disturbing the saturation of the tank at the beginning of elution. Nevertheless long periods of equilibration are undesirable when chemical reactions, such as oxidation or reduction of the metal salt, tend to take place on the paper.

Not all workers, however, have regarded complete saturation as necessarily desirable. A. LACOURT[72] has deliberately used different solvents for saturation and for elution, showing that it is often possible thereby to improve the separation. T. V. ARDEN[73] and J. A. LEWIS[17]

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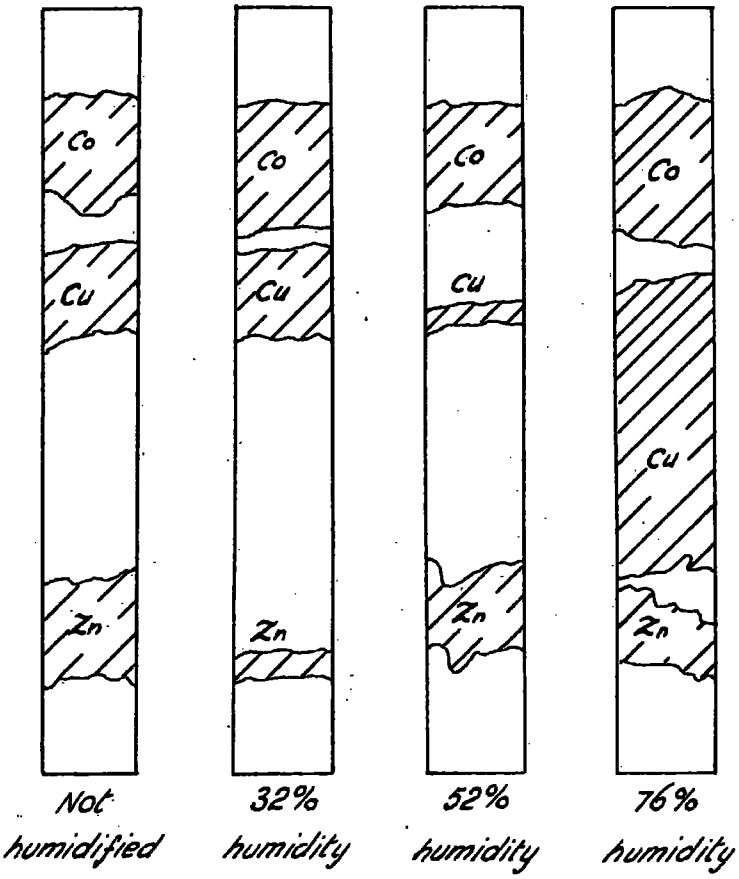

Fig. 11. Effect of humidification of paper on separation of Co, Cu, and Zn using a methyl ethyl ketone solvent (A. Lacourt[65])

found that in certain separations it is necessary to control the humidity of the tank during chromatography. S. V. VAECK[74] and A. LACOURT[65,75] have pointed out the effect of humidifying the paper prior to chromatography (Fig.11).

(g) Temperature at which a chromatogram is run

Temperatures of about 20°C are most often used. Elution takes place more rapidly at higher temperatures, but diffusion then causes greater spreading of the spots. Variation of temperature during elution is to be avoided as it may cause irregularities in the running of the chromatogram. This may be due to temporary unsaturation of the atmosphere or to the separating out of one of the components of a solvent.

(h) The method of chromatography used

Ascending chromatography is simpler but in many cases it is found that rather sharper separations can be obtained using the descending method. Radial chromatography[43] is said to produce rather sharper zones than ordinary chromatography.

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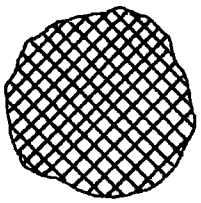
Appearance of spot

Graphs showing distribution of material in spot

Fig. 12a.



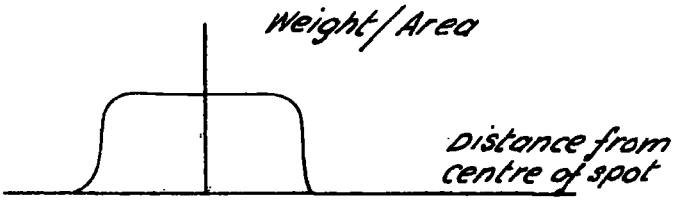
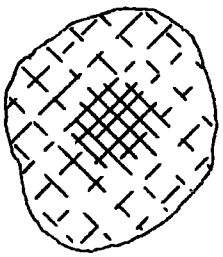


Fig. 12b.



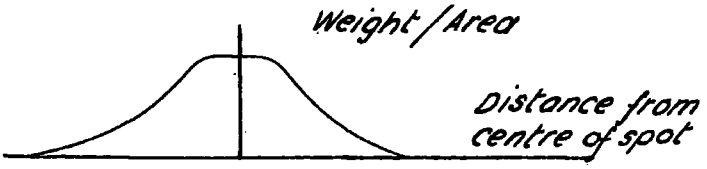
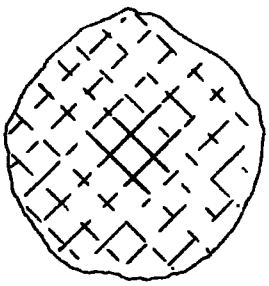
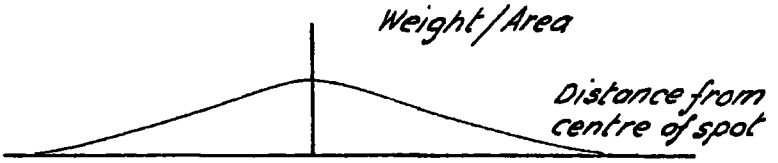


Fig. 12c.





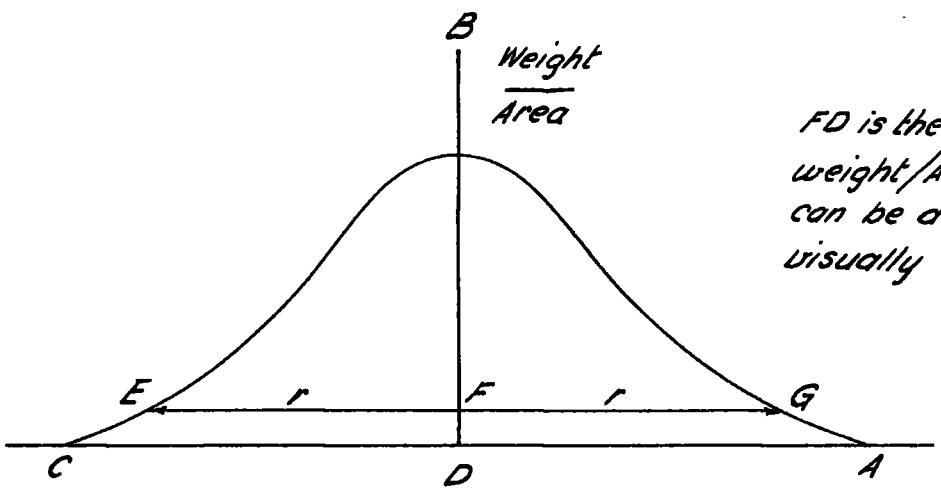


Fig. 12d. Graph showing the distribution of material in a chromatographed spot

§ 6

PHOTOMETRY ON PAPER: GENERAL PRINCIPLES

There are a large number of methods which have been used - or which could be used - for the estimation of paper chromatograms by photometry directly on paper. Some of the principles relating to these methods are considered in this section. § 7 is devoted to applications of these principles to individual methods.

(a) Modification of shape of spot during elution and general survey of methods

A uniform spot is normally obtained when the material is first placed on the paper, as shown in Fig.12a. The curve of weight per unit area versus distance from the centre of the spot will be as shown. As the spot spreads down the paper, it may, under suitable conditions, change in shape as shown in Figs.12b and c. If a number of spots of gradually diminishing concentration (but of the same volume) is placed on a sheet of paper side by side, and eluted and developed under identical conditions, then a series of spots of gradually diminishing colour density and size will be obtained. Each spot will, however, have a shape similar to that shown in Fig.12d. The area of the spot, which is dependent on r, may be used as a method of estimation. Alternatively, the distance DB (corresponding to the maximum colour density) or the product of DB and spot area may be measured. The distance DB is found experimentally by finding the transmittance T or reflectance R at the

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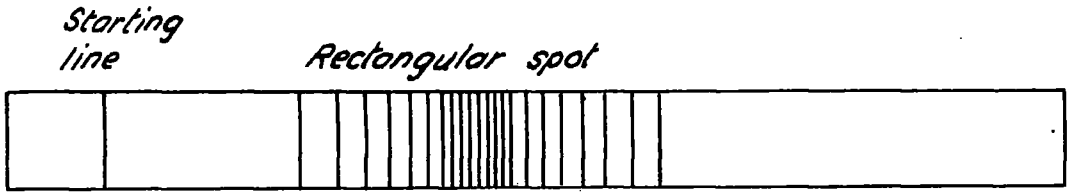

Fig. 13a. Appearance of strip chromatogram containing a single substance to be estimated.

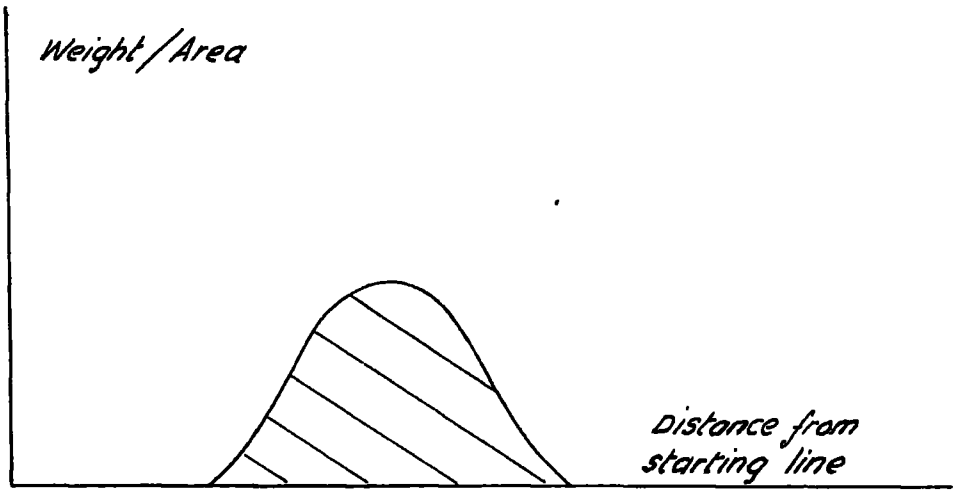

Fig. 13b. Curve showing distribution of material in the above spot

centre of the spot and using the relation

$$\text{Concentration} = f(T)$$
$$\text{or Concentration} = f'(R)$$

These relations are considered below on pages 30 - 35.

As might be expected, the most accurate methods are those which explore the whole of the spot. Here it is necessary to distinguish between two types of chromatograms:-

(i) Those run on comparatively narrow sheets of paper

Under suitable conditions, the final spots are as shown in Fig.13a. These will be referred to as 'rectangular' spots, which may be regarded as being made up of a number of narrow rectangular elements of equal colour density. The chromatogram may be measured by a scanning device, which employs a narrow slit of light a mm. or so wide and of length equal to the width of the paper. This is moved relative to the paper, and the transmittance or reflectance is measured continuously, or at intervals. The readings of transmittance or reflectance are used to measure the concentration by using the relation:-

$$\text{Concentration} = f(T)$$
$$\text{or Concentration} = f'(R)$$

referred to above, thus enabling a curve as shown in Fig.13b to be drawn. The total quantity of material in the spot may then be found by measuring the shaded area of the graph.

(ii) Those in which the final spot is a circle or ellipse

This type of chromatogram is obtained by placing a circular spot on a sheet of paper considerably wider than the diameter of the spot. The maximum width of the chromatographed spot is never as great as the width of the paper.

The exploration of the spot is necessarily more difficult here. It may be done by regarding the spot as being made up of a number of small squares each of uniform colour density as shown in Fig.14. The spot is scanned by a small square aperture in two dimensions, so that the whole of the area of the spot is covered. The total quantity of material is then found by integration. This procedure would be laborious if it were carried out manually, but with suitable instrumentation, it provides a practical and highly developed method, since the total quantity measured is not affected theoretically by the size, shape, or regularity of the spot.

The method in which the spot is scanned in only one dimension by a slit is, however, preferred by most workers on account of its simplicity. This method is often carried out on circular or elliptical spots as well as on rectangular ones. In such cases, the method is not so sound theoretically, since there is a variation in colour density within each element. Thus, the result will be subject to variations in the shape and size of the spot, and it is therefore desirable to restrict the method to estimating spots run under standard conditions. The length of the slit is usually made rather shorter than the width of the spot (Fig.15).

A further method of exploring the entire spot is to cut a mask to the size of the spot, and to pass through it a beam of light. Here, only a single reading is taken. This method can be used only when comparisons are made with other spots run under identical conditions, since the result will be affected even more by variations in the shape and size of the spot than in the method considered in the previous paragraph.

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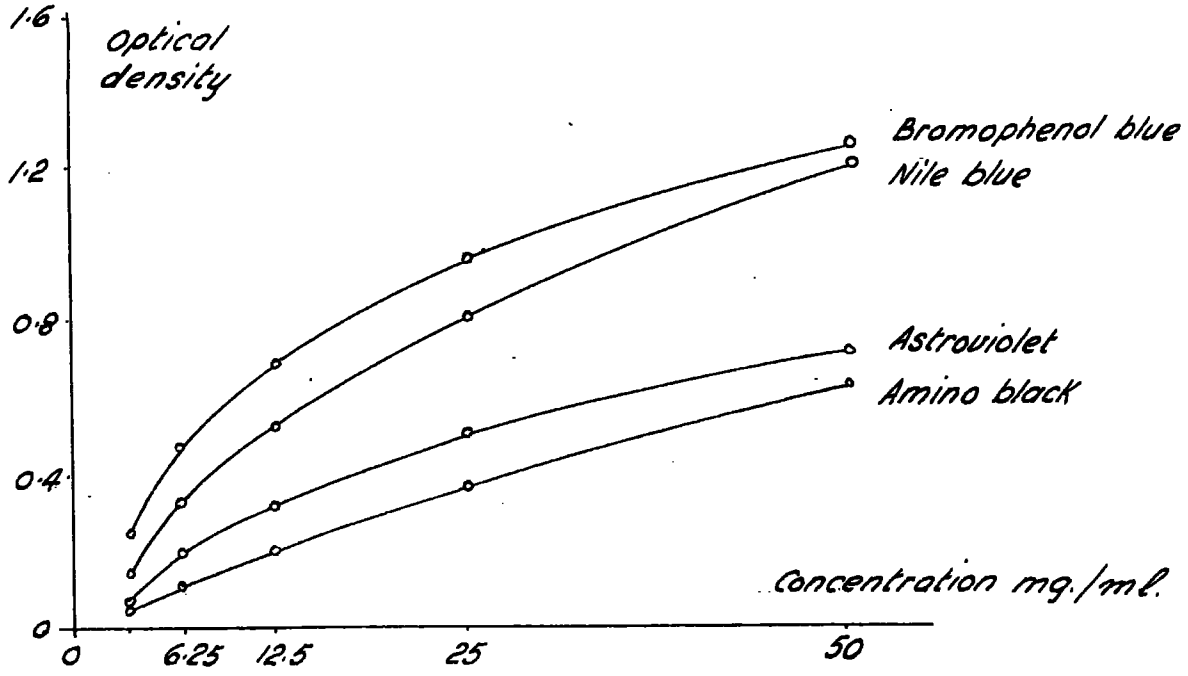

Fig. 16. Optical density curves for non-chromatographed spots using filtered light and transparent paper (P. Albers[76])

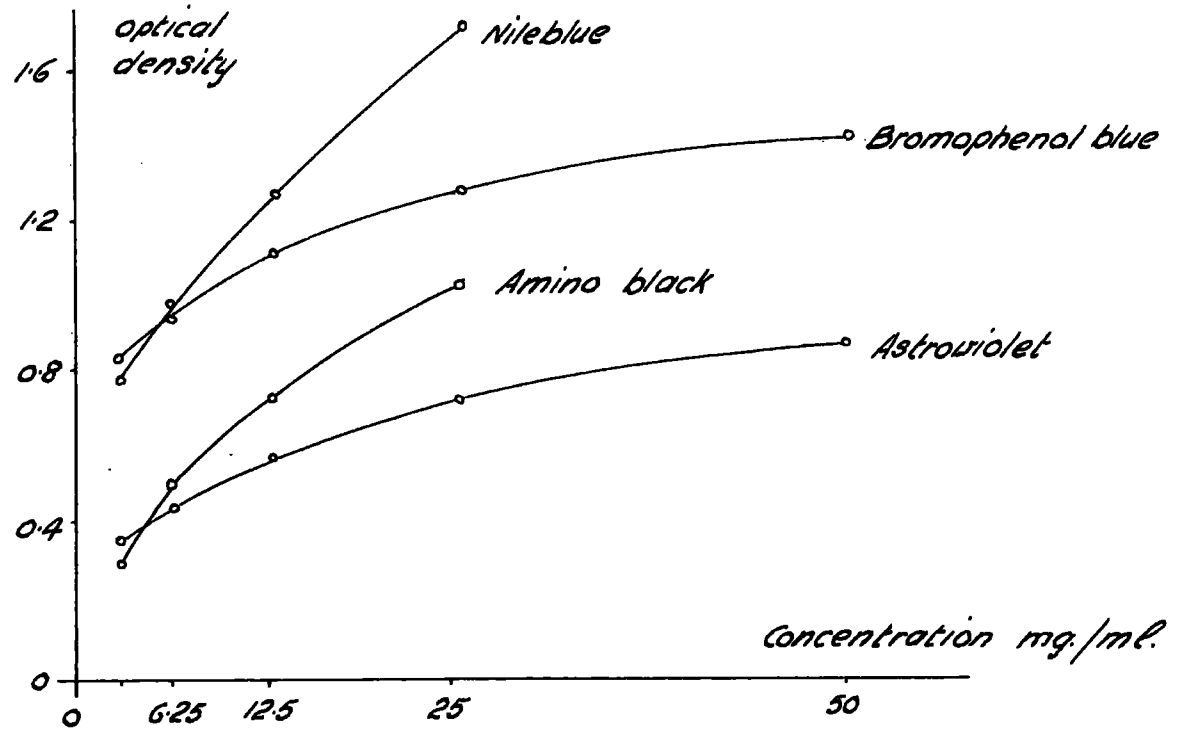

Fig. 17. Same as above but using non-transparent paper (P. Albers[76])

(b) Desirability of scanning

In principle, either transmission or reflection may be used for any of these methods, but the nature of the functions connecting the concentration of the material in the original solution with transmittance or reflectance are different. Unfortunately there is not a linear relation between either the transmittance and the concentration, or the reflectance and the concentration. If there were, a spot could be estimated by a single beam of light passing through the whole spot, regardless of the distribution of material within the spot. It is for this reason that scanning is necessary, except when comparisons are made between spots that have been eluted under identical conditions.

(c) Relation between transmittance and concentration for non-chromatographed spots - Desirability of rendering the paper transparent

Before considering the various methods of measuring chromatographed spots on paper, it is desirable to study the relation between transmittance and concentration for spots that have not been chromatographed. In solution, according to Beer's law, there is a linear relation between optical density (which is $\log \frac{1}{\text{transmittance}}$) and concentration. This linear relation normally holds, at least over a wide range, for spots on paper, but two important factors may interfere with it:-

(i) The use of white light or non-monochromatic light

Beer's law holds only for monochromatic light at an absorption maximum. Examples of curves obtained by P. ALBERS[76] (1959) of various dyes show clearly that Beer's law does not hold when non-monochromatic light (or light

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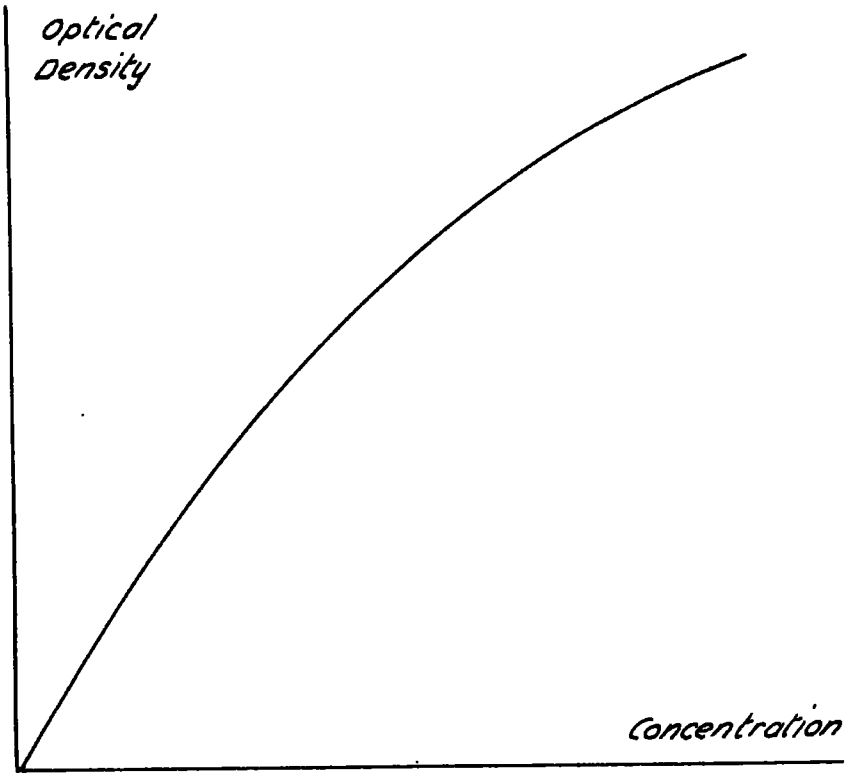

Fig. 18. Typical optical density curve for substance on non-transparent paper using monochromatic light

of the incorrect wavelength) is used (Fig.16).

(ii) The use of paper that has not been rendered transparent

It has generally been assumed that a linear relation does not hold unless the paper is rendered transparent, even when using monochromatic light of the correct wavelength. Albers compared the optical density curves obtained with transparent and non-transparent paper and showed that in the latter case the lines departed more from linearity than in the former case (Fig.17). Unfortunately, few workers have given optical density versus concentration curves for non-chromatographed spots, but it would appear that in general the curves obtained would be as shown in Fig.18. S. SAKURABA[77] (1955) obtained a curve of this shape for spots of copper and cobalt on dry paper using a spectrophotometer.

Straight line optical density versus concentration curves are not necessarily produced even when using transparent paper and working at the correct wavelength. E. M. CROOK[78,79] (1952) measured stained proteins (and also dyes alone) on transparent paper using a transmission densiometer fitted with a filter. Curves of the shape shown in Fig.19 were obtained, from which it became clear that there were considerable departures from Beer's law, even at quite low concentrations. The relation between photo-cell current and concentration was found to be hyperbolic rather than logarithmic and to follow approximately the equation

$$y = \frac{ac}{b + c},$$

where y is the photo-cell current,
c is the concentration,
and a and b are constants.

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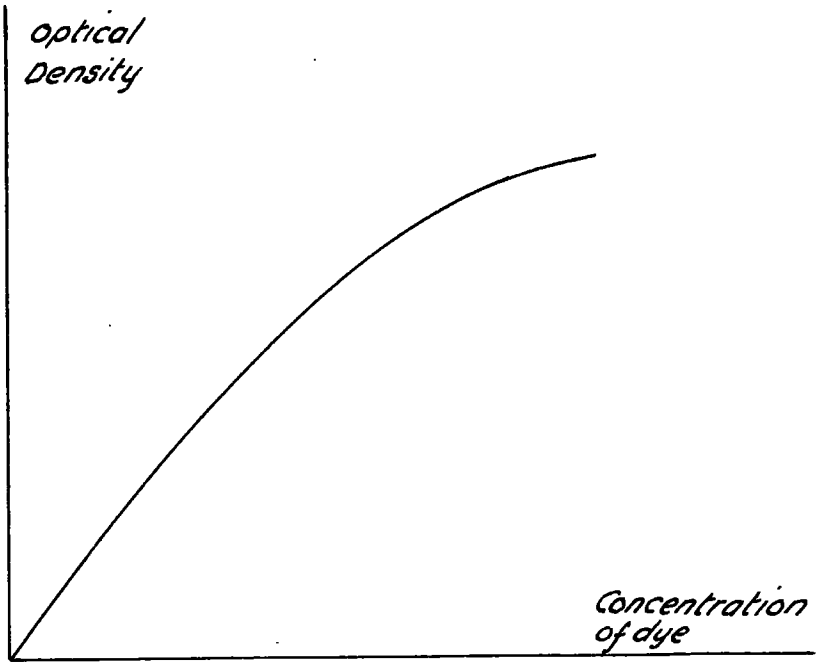

Fig. 19. Optical density curve of non-chromatographed dye (E.M. Crook [79])

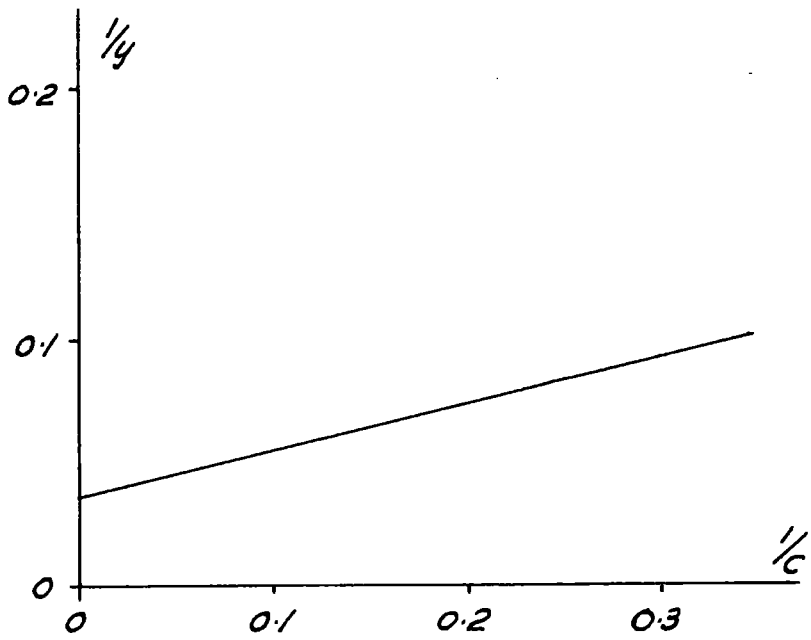

Fig. 20. Curve of 1/y versus 1/c (E.M. Crook [79])

Rearranged this became

$$\frac{1}{y} = \frac{b + c}{ac} = \frac{b}{a} \cdot \frac{1}{c} + \frac{1}{a} .$$

On plotting 1/y against 1/c, a straight line was obtained (Fig.20) although this linear relation held only over a limited range.

Many workers have preferred to render the paper transparent. This has been done in a variety of ways. W. GRASSMANN[80] (1950) employed anisole of refractive index 1·515 and also a mixture of paraffin and α bromonaphthalene adjusted to give the same refractive index as paper fibres, i.e. 1·51. A. L. LATNER[81] used methyl salicylate for the same purpose. I. N. ROSENBERG[82] applied 'Krylon Clarifier' followed by a plastic coating ('Krylon Plastic Spray') which had the advantage that the paper dried and could then be handled more easily. O. BASSIR[83] used a mixture of amyl alcohol and paraffin, and claimed that this treatment greatly reduced the non-homogeneity of the paper. A. LACOURT[84] used water (the paper was immersed in water) and found this a most suitable liquid, although the refractive index of water is much lower than that of paper fibres. S. V. VAECK[74] tried impregnating the paper with paraffin, paraffin oil, and α bromonaphthalene, but rejected these as, although the transparency of the paper was increased, the effect was also to increase the non-homogeneity of the paper. J. BARROLLIER[85] used a polyurethan resin 'herbopan' which dries to a colourless film of refractive index 1·54. A. PALACKY[86] employed silicone oils, perhaps on account of their very low surface tension and consequent rapid-spreading properties.

The advantages and disadvantages of rendering the paper transparent are:-

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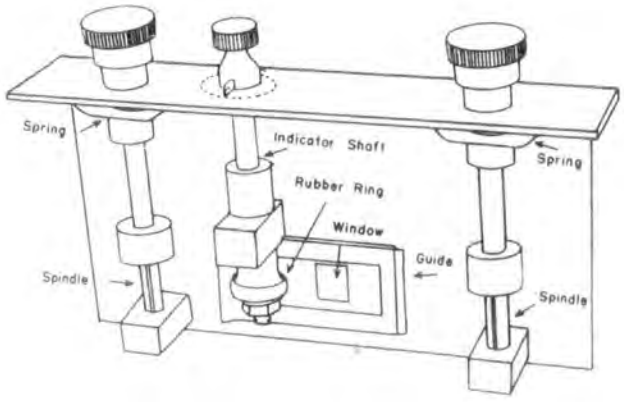

Fig. 21. Adaptor for scanning paper strips by transmission in a spectrophotometer (D.M. Tennent [88])

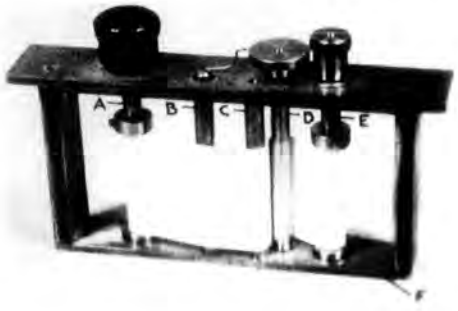

Fig. 22. Adaptor for scanning paper strips by transmission in a spectrophotometer (M. Beroza [89])

Advantages

(i) More light is transmitted. This is an important advantage when an insensitive photometer is employed.

(ii) Deviations from Beer's law are reduced.

Disadvantages

(i) It takes longer.

(ii) The procedure may be more subject to errors, as it may be difficult to exclude air bubbles.

(iii) It makes storage of chromatograms more difficult.

There does not appear to be any general agreement as to whether rendering the paper transparent decreases the non-homogeneity of the paper.

The properties required for a suitable reagent to render the paper transparent are:-

(i) A colourless non-volatile liquid of approximately the same refractive index as paper.

(ii) The liquid must not leach out the colour in the spot.

(iii) On immersion of the paper in the liquid, the transmittance should rapidly take up its correct value. The degree to which this happens probably depends on both the surface tension and the viscosity of the liquid.

No liquid is ideal for this purpose but, according to R. J. BLOCK[87], ordinary liquid petroleum (refractive index 1·43) is as satisfactory as liquids of exactly the correct refractive index. Two distinct methods, both of which are in general use, are employed for its application:-

(i) Sandwiching the paper with oil between two sheets of glass. This method may suffer from entrapped air bubbles.

(ii) Dipping the paper in oil and then allowing the paper to hang for some time before measurement.

Numerous transmission scanning adaptors for use in a spectrophotometer have been described. Two examples are shown in Figs.21 and 22 (D. M. TENNENT[88] and M. BEROZA[89]).

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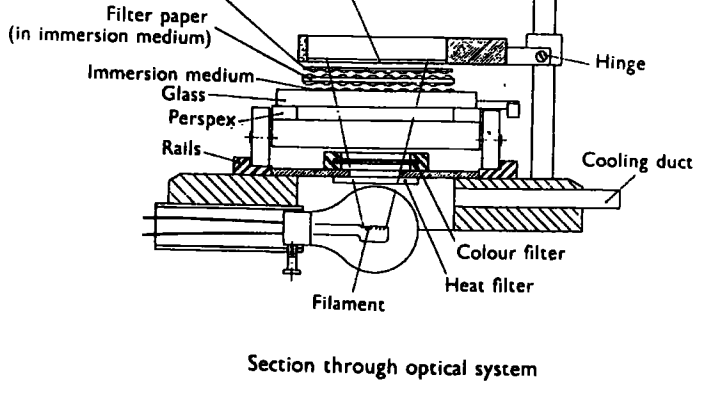

Section through optical system

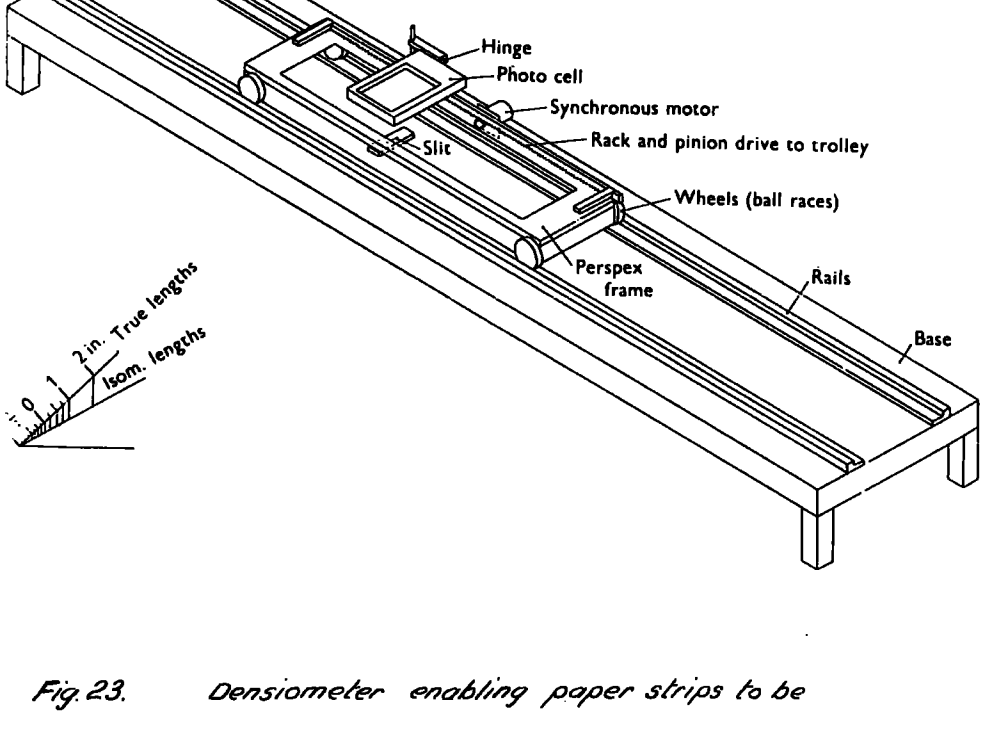

Fig. 23. Densiometer enabling paper strips to be scanned by transmission (E.M.Crook [79])

An example of a transmission scanning photometer employing filtered light is shown in Fig.23 (E. M. CROOK[79]).

(d) Relation between reflectance and concentration for non-chromatographed spots

The relation between the reflectance and the concentration of a coloured substance can be predicted from the theory of P. KUBELKA[90] (1931). F. A. STEELE[91] and D. B. JUDD[92] have commented on this theory and also S. V. VAECK[57], who used reflection measurements to evaluate spots. Reflection measurements on dry paper comply with the Kubelka-Munk law:-

$$R_\infty = 1 + K/S - \left[(1 + K/S)^2 - 1\right]^{\frac{1}{2}}$$

or $$K/S = (1 - R_\infty)^2 / 2R_\infty ,$$

where R_∞ = monochromatic reflectance of a material of such thickness that a further increase in thickness does not alter the reflectance reading,

K = absorption coefficient (fraction of incident radiation lost by absorption per unit thickness of material), and S = scattering coefficient (fraction of incident radiation lost due to scattering per unit thickness of material).

Assuming, as Vaeck did, that at least within certain limits, the coloured substance and the substratum material absorb and scatter light independently of one another, and that the ratio K/S is proportional to the concentration of coloured substance, then

$$kc = \left[\frac{(1 - R_\infty)^2}{2R_\infty}\right]_{\text{total}} - \left[\frac{(1 - R_\infty)^2}{2R_\infty}\right]_{\text{paper blank}}$$

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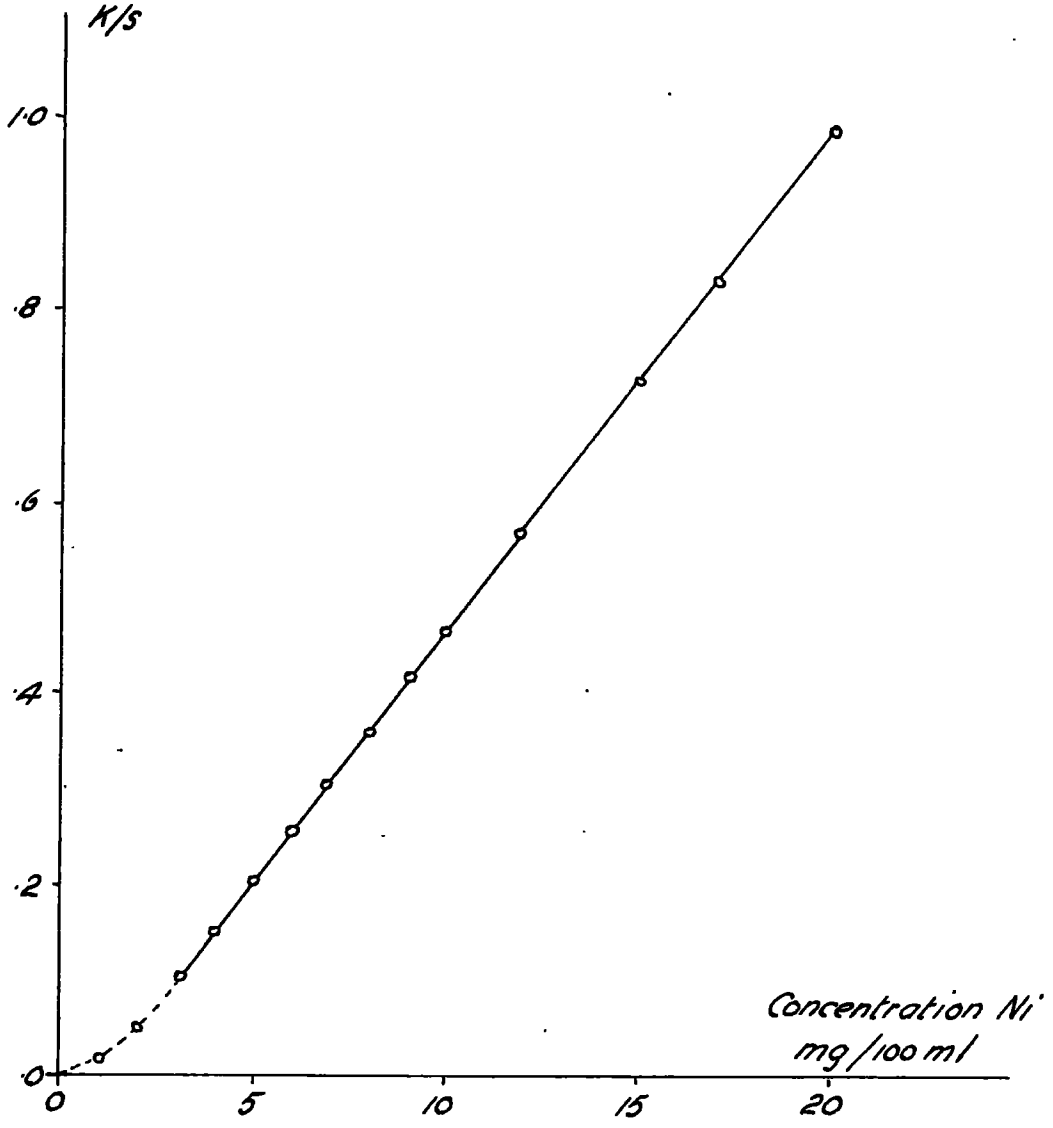

Fig. 24. Curve of K/S versus concentration curve for nickel (S.V. Vaeck [57])

where c is the concentration of coloured substance and k is a constant.

Vaeck argued that the Kubelka-Munk law would not be expected to apply, since in practice:-

(i) The readings of reflectance are made against a paper blank as standard.

(ii) The coloured material is not opaque and part of the reflected light comes from the white background.

(iii) The colour may not be evenly distributed throughout the paper at all concentrations.

However that may be, when a graph of K/S versus concentration was drawn[57], a straight line was obtained over a wide range of concentration as shown in Fig.24. S. SAKURABA[77,93] (1955) also obtained straight lines by plotting concentration versus K/S for copper and cobalt complexes on paper. D. B. JUDD[94] gave a table enabling the K/S value to be obtained directly from the reflectance reading measured on the photometer.

(e) Comparison of suitability of transmission and reflection

(i) Instrumental convenience

It is easier to design an instrument for transmission measurements than for reflection measurements, especially when scanning has to be carried out. This is certainly the most important reason for the overwhelming preference for transmission. Transmission readings are made by interposing the paper between the light source and the photo-cell (Fig.25). A spectrophotometer can be readily adapted for this purpose. For reflection, the collection of the light rays reflected by the paper on to the photo-cell is much more difficult (Fig.26). Reflection attachments are available for several spectrophotometers, such as the Beckmann and the

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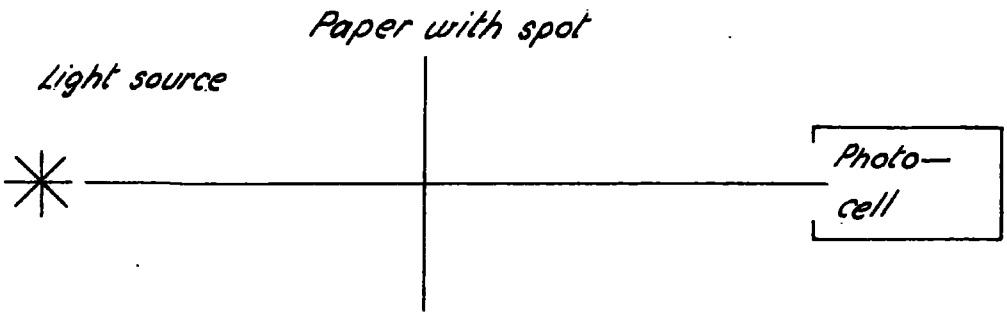

Fig. 25. Principle of measurement of spot by transmission

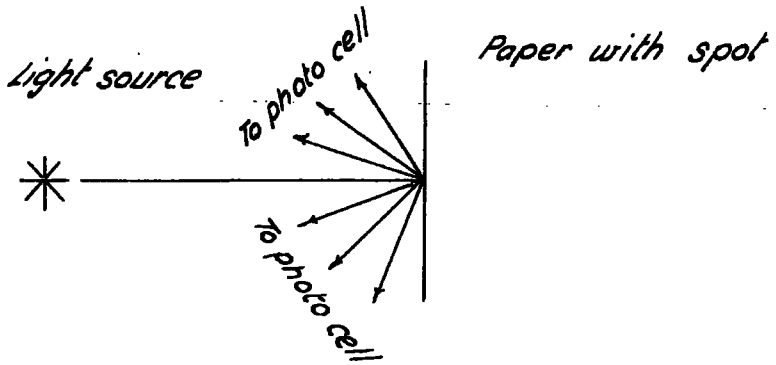

Fig. 26. Principle of measurement of spot by reflectance

Uvispek, but these do not permit scanning. The Joyce-Lobel photometer enables scanning to be carried out by reflection, but does not use monochromatic light.

(ii) Sensitivity and accuracy

E. H. WINSLOW[95] showed that estimations based on transmission for copper, developed with α-benzoin oxime, are more sensitive than those based on reflection.

A point of considerable interest is the establishment of the relative accuracies of transmission and reflection methods. Since the non-homogeneity of the paper is a most important factor in reducing the accuracy of quantitative estimations directly on paper, it might well be thought that reflection would be superior. A coloured spot on paper certainly appears uniform to the eye by reflected light, whilst it appears quite non-uniform when held up to the light. Perhaps the superiority of reflection measurements, if this superiority does in fact exist, would be expected to be most apparent when a small area of paper, say a few sq. mm., is considered. However, neither C. W. AYERS[96], who used the Joyce-Lobel scanning reflection apparatus for estimating non-chromatographed spots of iron, cobalt, and nickel, nor Vaeck claimed any superiority for reflection measurements over those of transmission. S. SAKURABA[77] also investigated the spot test analysis of copper and cobalt by spectrophotometric measurements using both transmission and reflection. To judge from the original Japanese article, with English summary, it would appear that estimations based on transmission measurements were somewhat more sensitive than those based on reflection. The non-homogeneity of the paper was said to be responsible for unexpected errors in transmission readings but,

notwithstanding this, the average error appeared to be less by transmission than by reflection. E. M. CROOK[79] stated that transmission measurements were greatly to be preferred because of the variations in surface texture of the paper and the differences in degree of penetration of the colour throughout the paper which adversely affected the accuracy of reflection measurements.

(f) Comparison of suitability of a spectrophotometer with that of a photometer employing white or filtered light

Since both Beer's law and the Kubelka-Munk law apply strictly only for monochromatic light, it follows that a spectrophotometer is to be preferred to a photometer employing white or filtered light. According to R. J. BLOCK[97], closer approximations to Beer's law are obtained with monochromatic light from a spectrophotometer than with even the relatively narrow bands available with modern interference filters. Furthermore, spectrophotometer measurements are much more sensitive than white light measurements.

Nevertheless, it must not be thought that straight line optical density versus concentration curves are essential for accurate analysis. If the spots are to be scanned, straight line curves are certainly a great convenience, since the area under the optical density scanning curve is then proportional to the spot content. When the optical density versus concentration curve is not a straight line, it is necessary to compensate for the deviations by calibration[97]. Indeed, many workers have paid no attention whatsoever to the optical density versus concentration curves for non-chromatographed spots, and have been content to use some empirical relation between the area under the optical density scanning curve of the chromatographed spot and the spot content.

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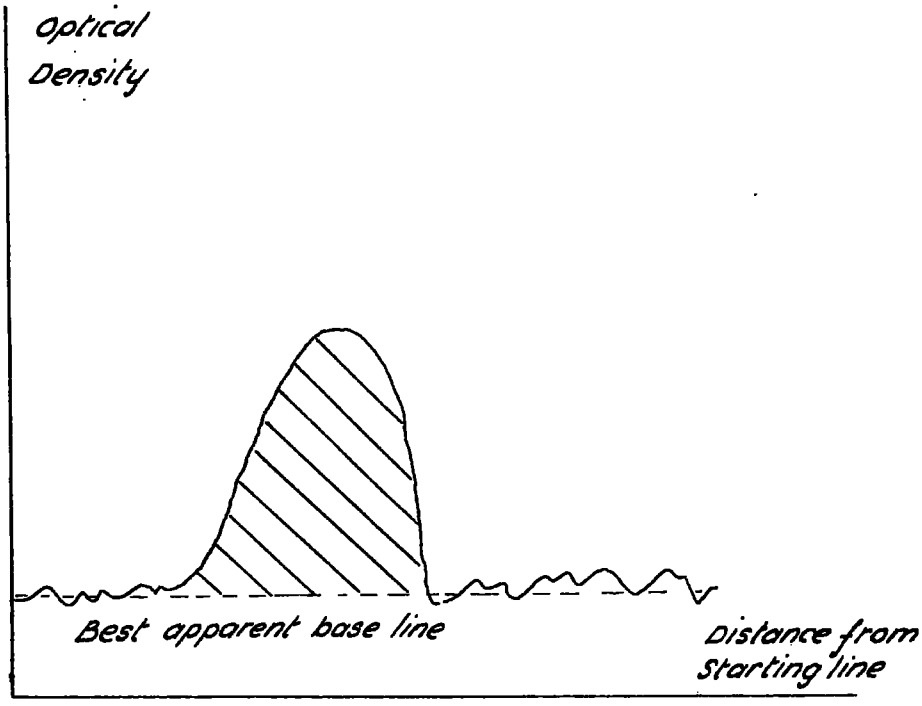

Fig. 27. Method of drawing the best apparent base line

(g) Method of using blanks

Whichever method of estimating the substance is used, the photometer reading must be related to that of the white paper or the blank paper. In using scanning methods, this is done by drawing the best apparent base line (Fig.27).

In the case of single reading methods of estimation, the instrument is set to zero optical density (100% transmittance):-

either (i) on a sheet of white filter paper from the reel,

or (ii) on a piece of filter paper sprayed with the developer. This piece is usually taken from an area adjacent to the spot which is being measured,

or (iii) using a 'blank spot'.

The blank spot technique is to place several spots on the paper each containing metals in acid solution and also a spot of acid of the same volume but without any metal. After elution and development, the position of the paper where a spot would have been obtained had a metal been present, is termed the blank spot.

Accurately reproducible blanks are essential, as stressed by A. LACOURT[98]. Of the above methods, that using a blank spot is the soundest, but method (ii) may give equally accurate results. Method (i) is satisfactory only if the developer is colourless.

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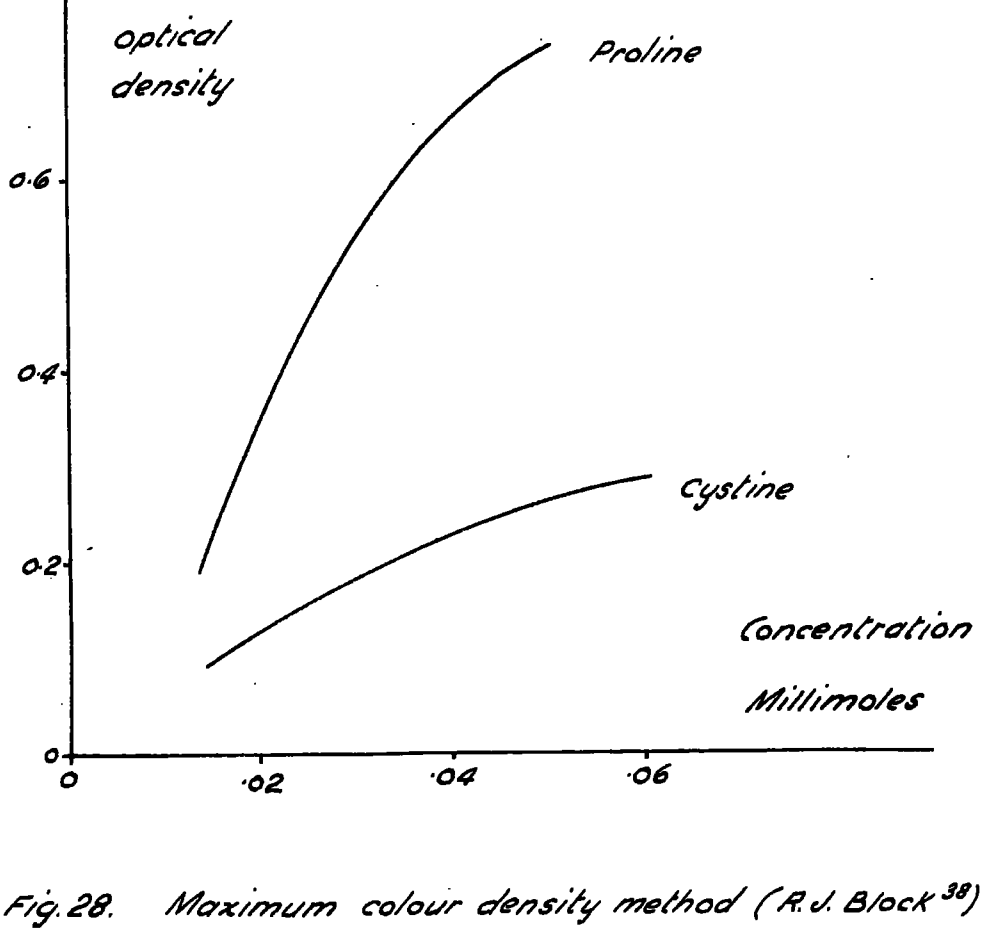

Fig. 28. Maximum colour density method (R. J. Block [38])

§ 7

PHOTOMETRY ON PAPER: APPLICATIONS

This section is devoted to the practical application of various methods of estimation directly on paper.

(a) Maximum colour of spot

It might be thought that any method involving a single reading, which does not explore the whole spot would produce only an approximate result, but this is not necessarily the case, provided comparisons are made between standards and unknowns which are run on the same sheet of paper under identical conditions. It is essential, however, that reasonably uniform spots be obtained, but the difficulty in producing these, imposes a severe limitation on the method. According to the theory of R. C. BRIMLEY[34], to which reference has already been made (page 15), there should be a linear relation between concentration in the original spot and the maximum weight per unit area in the chromatographed spot. Even if this theory is not correct, there must be some relation between these two factors which may be made the basis of a method of estimation.

This method was used by R. J. BLOCK[38,99] (1950) in the estimation of amino acids. A transmission densiometer was fitted with a mask of 5 x 5 mm., and the chromatogram was pulled slowly across the beam of light. No filter was employed. The deflection of the galvanometer was noted at each peak and the concentration of the substance was then calculated using the relation

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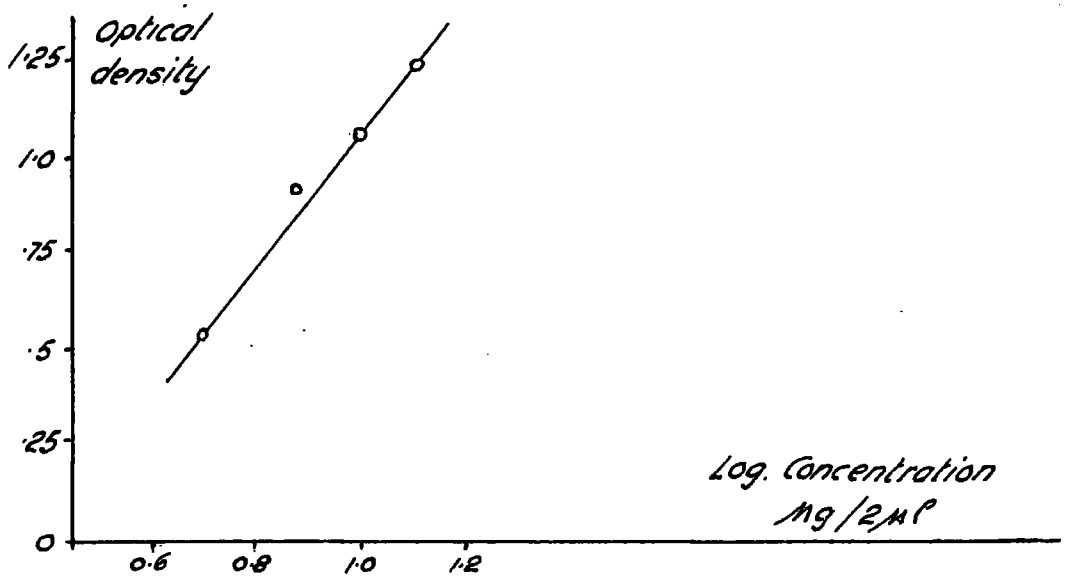

Fig. 29. Maximum colour density method applied to a sugar (E. F. McFarren[102])

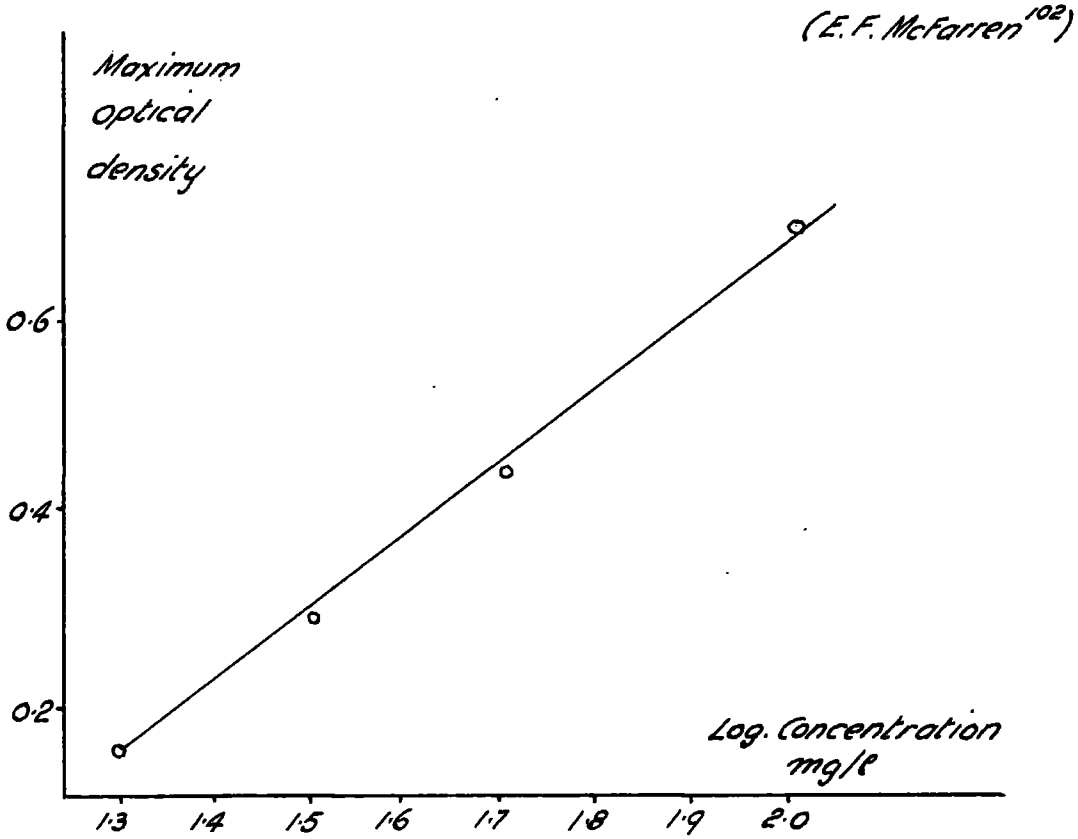

Fig. 30. Maximum colour density method applied to an amino acid (H.C. Ehrmantraut[103])

Concentration of material in entire spot	$\propto$	Maximum optical density of each spot

This linear relation applied only when the initial spot size was kept constant. The curves obtained are shown in Fig.28. This method was found to give more accurate results than the measurement of spot areas.

A. R. PATTON[100] (1951) and F. X. GASSNER[101] (1952) used a method similar to that of Block and claimed that, under properly controlled conditions, the accuracy was comparable to that obtained by scanning and very much more rapid.

E. F. McFARREN[102] (1951) using a 5 mm. diameter aperture, and H. C. EHRMANTRAUT[103] (1954) using a beam of light from a spectrophotometer that fell well within the spot, also applied the maximum colour method. In both cases straight lines were obtained on plotting optical density versus the logarithm of the spot concentration (Figs.29 and 30).

The maximum colour method was used for metals by S. V. VAECK[74] and this application will now be described in some detail. A mixture of copper, cobalt, and nickel was studied although only the cobalt was actually estimated quantitatively. Two methods of estimation were used: the maximum colour method, which is now under consideration; and the measurement of total colour by scanning with a slit. For convenience these will now be considered together.

Determination of cobalt (S. V. VAECK[74])

(i) Production of initial spots

The metal salts were dissolved in 3 N hydrochloric acid, which was found to give very uniform initial spots. Volumes of about 10 μl which gave spots about 15 mm. in diameter were found to be convenient and were applied by an accurate micro-pipette.

Facing P. 41

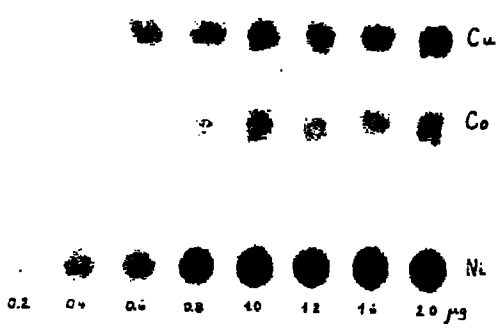

Fig. 31. Appearance of chromatogram of Ni, Co, and Cu (S.V. Vaeck[74])

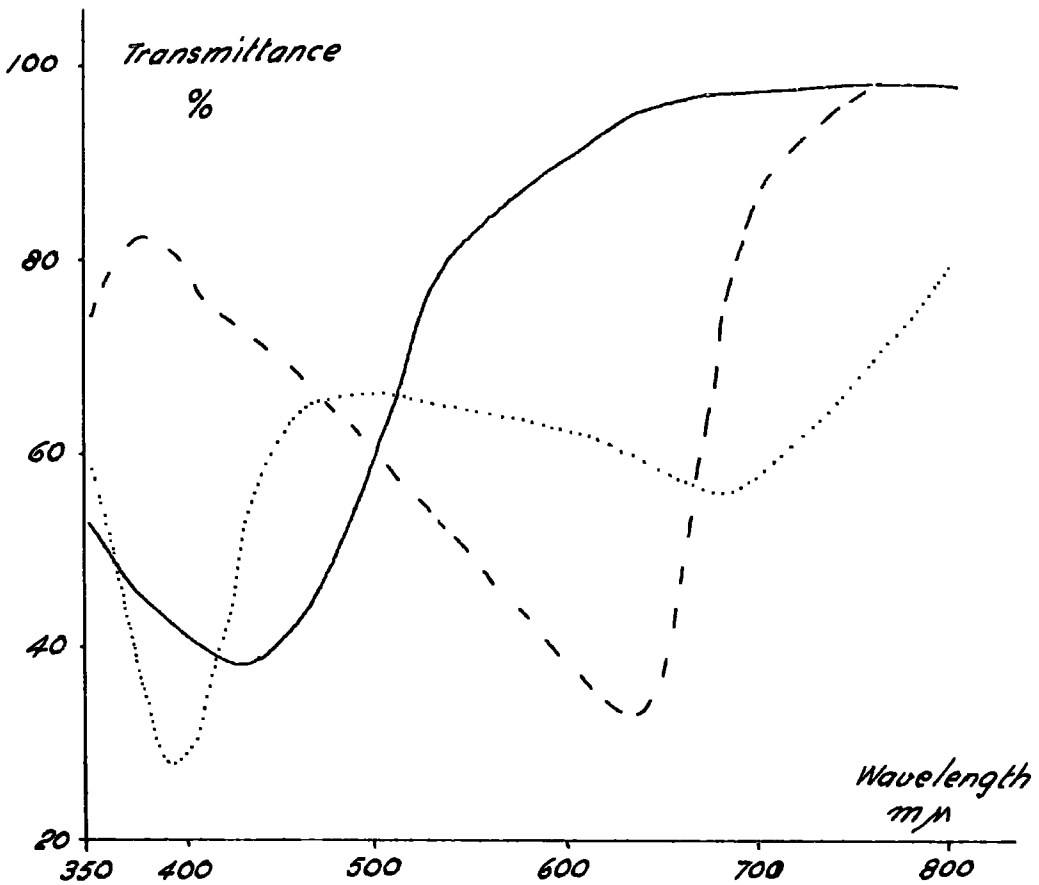

Fig. 32. Transmission curves of spots containing amounts of Ni, Co, and Cu. (S.V. Vaeck[74])

(ii) Chromatography

Ascending chromatography on paper 23 x 23 cm. was employed. Eight spots could be run side by side (4 standards and 4 unknowns). The eluting solvent used was n-butanol 30 ml., acetone 56 ml., hydrochloric acid (25%) 12 ml., and acetylacetone 2 ml. It was found important to control the moisture content of the papers prior to chromatography. This was done by hanging the papers for one hour in a constant humidity cabinet, containing a tray of saturated ammonium nitrate solution and a fan. The papers were transferred as quickly as possible from the cabinet to the chromatography jars, where they were eluted for 3 hours. The appearance of the chromatograms is shown in Fig.31.

(iii) Colour production

0·03% alcoholic solution of rubeanic acid containing 5% concentrated ammonia was sprayed evenly on both sides of the chromatogram. The colours produced were stable when protected from daylight. The spectral transmission curves for the metal complexes were determined, as shown in Fig.32. The absorption maximum was found to be at 422 mμ for cobalt. This wavelength was used for all subsequent measurements.

(iv) Colour measurements

The paper was cut into strips in the direction of the solvent flow and each one was scanned. Both a photometer and a spectrophotometer were used. The photometer had a high sensitivity enabling narrow band filters to be employed. It was provided with a mechanism enabling the paper to be advanced mm. by mm. and had a slit of 1 mm. x 15 mm. When using the spectrophotometer, the paper was advanced 2 mm.

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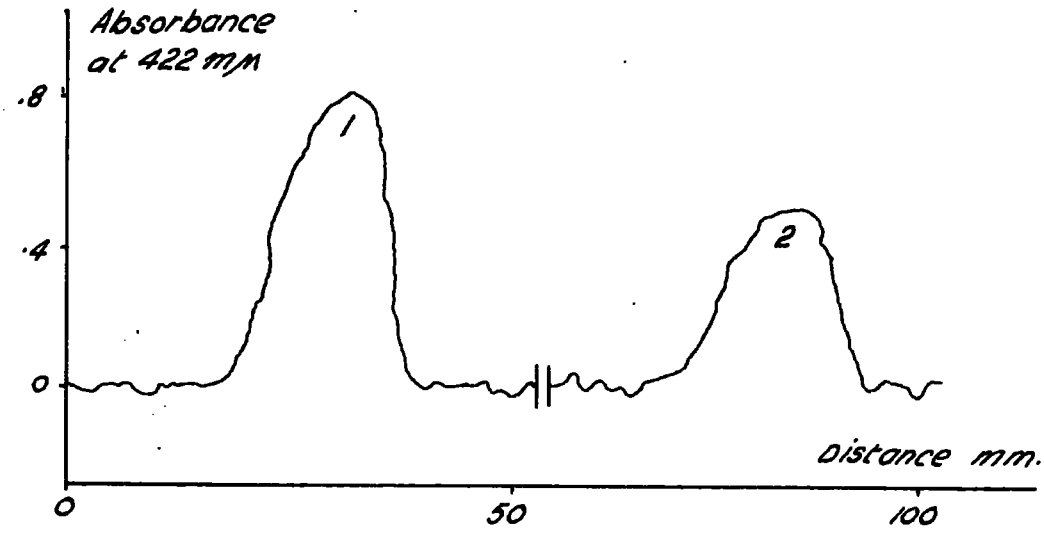

Fig. 33. Absorbance curves obtained (1) with 2 μgm Cu (2) 0·8 μgm Co (S.V. Vaeck[74])

at a time. In both cases, the paper was scanned dry. A typical curve is shown in Fig. 33. (This was with the spectrophotometer: similar curves were obtained using the photometer). Readings with the densiometer were rather less precise, but much more rapid.

Calibration curves for various concentrations were drawn both for area under the transmission curve and for maximum height of the curve. These are shown in Fig.34. In order to compare the precision of the two methods a series of six chromatograms was prepared, each containing eight identical spots. It was found that over the range 4 to 20 mg.Co/100 ml., the coefficient of variation of a single measurement was about 7% for height and 6·5% for the enclosed area. Vaeck argued that "---- the average calibration curve for the area is nearer a straight line than the curve for the height, so that the coefficient of variation of a single measurement expressed in % Co increases from 7 to 10% (from 4 to 20 mg.Co/100 ml.) when using the height and only from 6·5 to 7·5% when using the total area". This reasoning does not appear correct, if the calibration curves shown are typical ones. The fact that the area calibration curve is nearer a straight line is of little importance. The question of importance is the change in measured concentration brought about by variations of 7% for height and 6·5% for area. At the concentration of 10 mg.Co/100 ml., it is clear from the graph that these variations of 7% for height and 6·5% for area will produce almost the same change in measured concentration. It would appear therefore, that the two methods are of almost the same precision in this particular case.

A. BEVENUE[104,105] (1958) used a maximum density method which is of some interest as the readings were taken by

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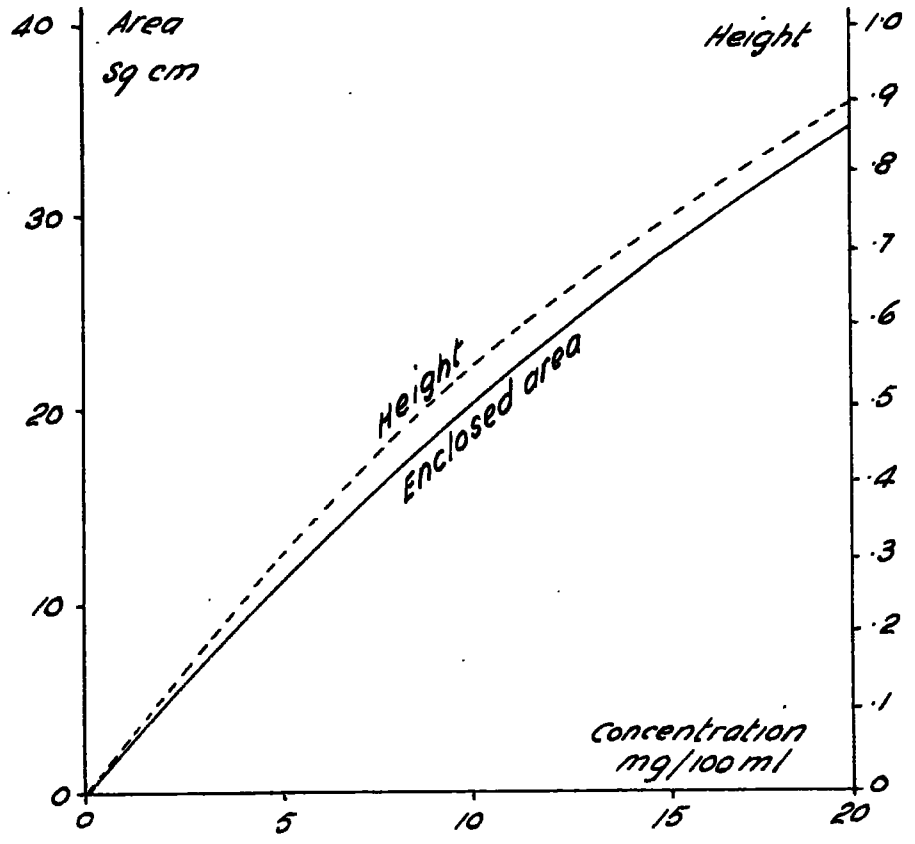

FIG. 34. Calibration curves for Co obtained from chromatographed spots containing 0-2 μgm Co (S.V. Vaeck [74])

reflection. Spots of sugars were eluted on large rectangular sheets of paper side by side, and each spot was scanned by reflection. The minimum reflectance was noted, and it was found that there was a linear relation between the logarithm of the concentration and the reflectance. This linear relation held, however, only over a very limited range.

(b) Maximum colour x area of spot

R. J. BLOCK[38,106] (1948) working on amino acids showed that there was a linear relation between the concentration of the original spot and the maximum optical density of the chromatographed spot. This linear relation did not hold, if, as was occasionally desirable, initial spots of varying sizes were used. In these cases, however, it was possible to make use of a linear relation between the concentration of the substance and the area of the spot multiplied by the maximum optical density. This method is particularly valuable when compact spots cannot be obtained. D. BOLLING[107] (1949) also used this method in estimating amino acids.

(c) Total colour of spot - single reading over the whole area of the spot

[This is one of three methods which are employed in estimating the total colour of the spot: the other two are considered in paragraph (d) on page 47 and paragraph (e) on page 49.]

Since neither the relation between concentration and transmittance, nor the relation between concentration and reflectance is a linear one, it follows that neither transmittance nor reflectance can measure the total concentration of a substance in a chromatographed spot by this method, unless all other factors are rigorously controlled. For example, two spots that have been eluted for different times

will have a different surface distribution on paper: one may be compact and the other diffuse. If measured by a beam of light extending over the whole area of the spot, they will certainly not have the same reflectance or transmittance. Strictly, in this connection, the terms transmittance, optical density, and reflectance have no meaning and should be replaced by apparent transmittance, apparent optical density, and apparent reflectance; but as this terminology is not in general use it will not be applied here. However, comparisons can be made between the (apparent) optical density and the concentration of different spots provided the spots are run under the same conditions. It is, therefore, important to study carefully the factors affecting the running of a chromatogram and to standardise them closely.

L. B. ROCKLAND[108] (1949) estimated amino acids by this method. After elution and development, the paper containing the spots was placed in a holder provided with an aperture of a size just sufficient to enclose the entire area of the spot. The colour intensities were read on a photo-electric colorimeter. The concentrations were estimated from standard curves drawn from plots of transmittance versus concentration of amino acid. Later L. B. ROCKLAND[109] developed this method further and emphasised the advantages of carrying the work out on a reduced scale, using small sheets of paper and small initial spots, thus greatly reducing the time taken for elution. A special adaptor was described, employing three circular masks and three elliptical masks, any one of which could be accurately centred over the spot. On plotting absorbance against initial concentration, straight lines were obtained.

S. V. VAECK[57,110] (1953) used this method in the estimation of nickel, using reflectance spectrophotometry, but only for spots whose R_f value is almost zero. Uniform initial spots were obtained by making up solutions of the metals in 3 N hydrochloric acid. In the spectrophotometer used, the aperture had a diameter of 25·5 mm. It was found that in order to produce spots of this diameter, 0·03 ml. of the initial solution had to be applied to the paper. Within limits, the colour intensity per unit area was almost independent of the volume placed on the paper, so that no accurately calibrated micro-pipettes were needed. About 0·045 ml. of the initial solution was applied and allowed to dry at room temperature, protected from draughts. Six spots (one blank, three standards, and two unknowns) were applied to the starting line of a rectangular sheet of paper, and the chromatograms were eluted by ascending chromatography at room temperature for about ¾ hour. The nickel hardly moved at all (R_f = 0·03), while copper and the majority of the other metals, travelled a considerable distance and were completely separated from the nickel. The nickel spots were scarcely distorted. The dried chromatogram was sprayed with 0·05% alcoholic rubeanic acid containing 5% concentrated ammonia, using a spray gun operated by nitrogen at 1 atmosphere pressure. Before spraying, the chromatogram was placed for a few seconds in ammonia vapour. The spots were measured in a Beckmann spectrophotometer at the absorption maximum of 625 mμ using a background of eight layers of white filter paper. The blank spot was used as the standard. The K/S values (see page 34) were obtained from tables from the reflectance reading, and were plotted against concentration to give a straight line, as shown in Fig.24 facing page 35. It should be noted that a fresh calibration curve had to be

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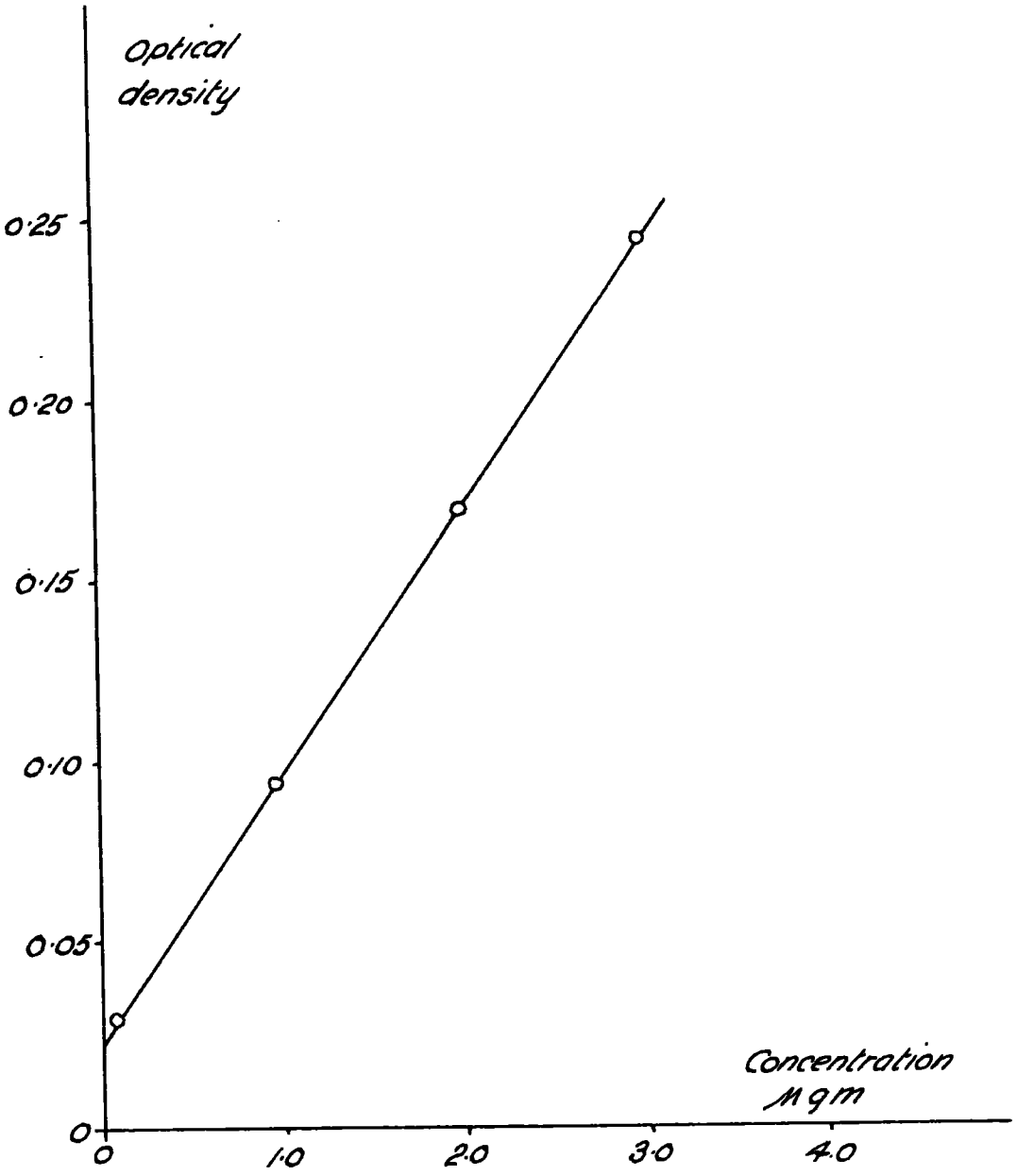

Fig. 35. Calibration curve for chromatographed copper, developed by rubeanic acid (A. Lacourt [114])

drawn for each chromatogram, and to enable this to be done three standards were placed on each chromatogram. If these did not lie very nearly on a straight line on the K/S versus concentration curve, this indicated that something had gone wrong and the chromatogram was rejected.

A. LACOURT[111-114] (1955) estimated cobalt and copper on paper by transmission spectrophotometry. The piece of paper containing the developed spot was placed with a few drops of water between the wall of the usual spectrophotometric cell and a 'prismatic calibrated glass adaptor'. Air bubbles were quickly eliminated and the optical density was then read in the spectrophotometer. A point of special interest is that the readings were not taken at the absorption maximum, but at a wavelength at which the absorption of the complex differed most from that of the developer. This reduced errors due to variations in density of the developer on the paper. In this method a beam of light of large cross sectional area was used so that the irregularities in the paper, which are so important on scanning with a narrow slit, did not show up. The reproducibility of the readings was very high, and the readings were said to be unaffected by the position of the spot on the paper. The method could be applied both to non-chromatographed and chromatographed spots, and its accuracy was stated to be ½ - 1%, within the range 0·1 - 3 μg. of cobalt and copper. A calibration curve is shown in Fig.35.

This method was later compared by A. LACOURT[115] (1956) with three other methods:-

(i) Absorptiometric estimation of spots after elution.

(ii) Direct photometric estimation by scanning in white light.

(iii) Direct titrimetric estimation of spots on the paper.

The advantages of direct spectrophotometric estimation in accuracy and saving of time were emphasised, and it was pointed out that more accurate determinations could be carried out on chromatographed spots than on non-chromatographed spots.

(d) Total colour of spot - scanning the spot by a slit, in one dimension

This method explores the whole spot and it would therefore be expected that the shape of the spot would be much less important than in the methods so far described. In the case of perfect rectangular spots on thin sheets of paper (see page 28), the results should be independent of the shape of the spot, but in the case of circular or elliptical spots this will not be so, as the density of colour varies along a given elemental strip. The method has been widely used in the estimation of amino acids and other organic substances. The first account of this method was that of R. J. BLOCK[106] (1948), in which strips were scanned by a transmission densiometer. The areas under the distribution curves were compared with those obtained from standards run side by side with the unknown. This method was further developed by H. B. BULL[64] (1949), R. R. REDFIELD[116] (1952), and numerous other workers. Generally, the area under the optical density distribution curve was taken as being linearly related to the concentration of substance in the original spot, regardless of whether the paper was rendered transparent or not and of whether monochromatic or non-monochromatic light was employed. The justification for this was that the errors so produced were cancelled out, when standards were run and measured in the same way. Certain workers have, however, considered these factors critically.

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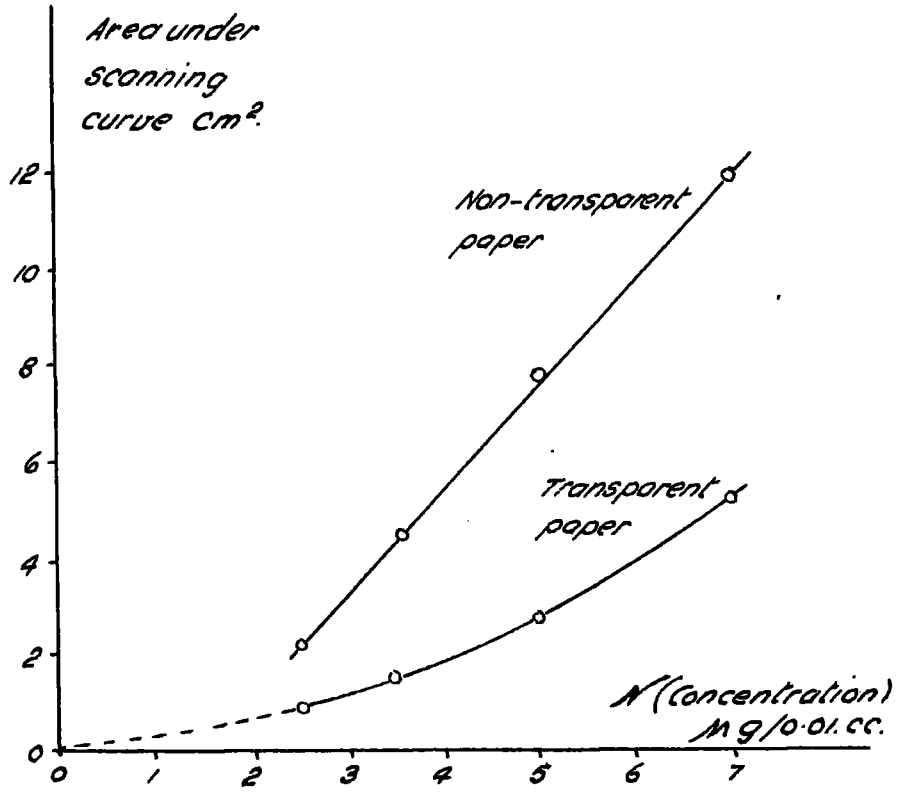

Fig. 36. Calibration curves for chromatographed mannose obtained by P. Albers[76] (Graph redrawn by present writer to include origin. Curve extended by dotted line to origin)

In particular P. ALBERS[76] (1959), whose work on non-chromatographed spots has already been mentioned (page 30), found that if both non-monochromatic light and non-transparent paper were used and if the chromatograms were scanned by transmission, the area under the curve was linearly related to the square root of the initial concentration. It seems clear, however, that this linear relation cannot apply at low concentrations. But if the paper was rendered transparent, no straight line was obtained, as shown in Fig.36. Recently B. M. JOHNSTONE[117] (1959) described an automatic recording transmission densiometer using a fluorescent lamp as a light source giving even illumination and no heat, thus enabling it to be mounted in contact with the paper. The densiometer was used in estimating amino acids, and it was found that the logarithm of the area under the transmission curve was proportional to the logarithm of the initial concentration.

Few attempts have been made to apply the method of scanning by a slit to metals. Perhaps the most important example is the work of S. V. VAECK[74] (1955) which has already been considered on page 40. Another important example is that of C. BERGAMINI[118] (1954) who applied the method to metals that had been separated by radial chromatography. Pb, Ag, Cu, Cd, Co, Ni and Tl were estimated. The most satisfactory solvent for elution was found to be 0·5 g. benzoylacetone, 50 ml. butanol and 50 ml. 0·1 N nitric acid. After development, the paper was rendered transparent by impregnation with anisole or a mixture of paraffin and α-bromonaphthalene. The instrument was set to zero on a point of the paper outside the ring, and then the paper was scanned along two orthogonal diameters. The type of curve produced is shown in Fig.37. The curves were in each case

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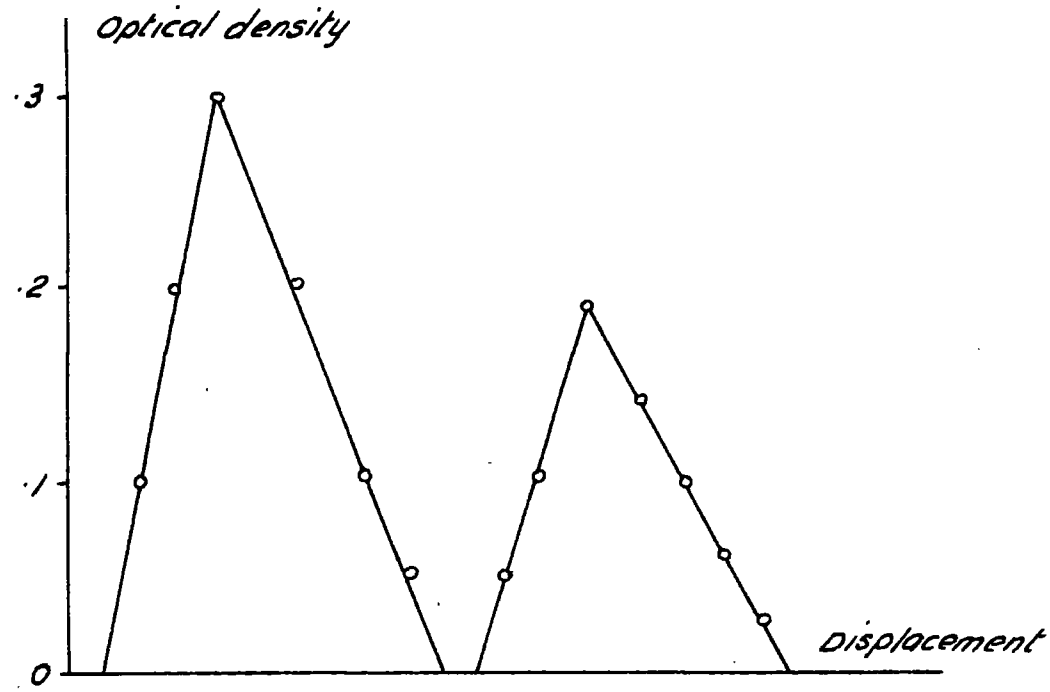

Fig. 37. Typical scanning curve for metals separated by radial chromatography (C. Bergamini[118])

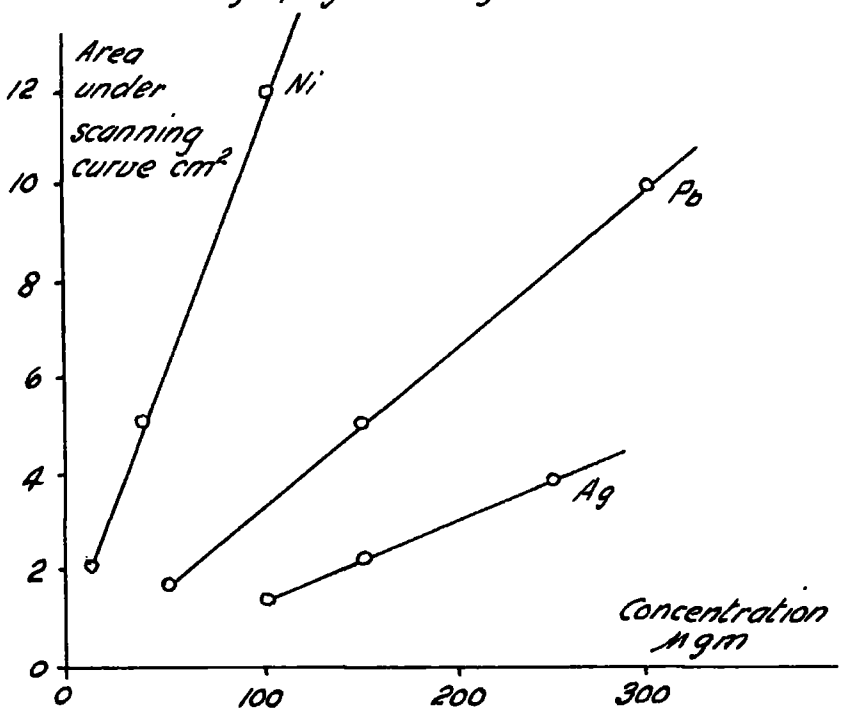

Fig. 38. Calibration curves for metals separated by radial chromatography (C. Bergamini[118])

triangular in shape and the area of these was taken as a measure of the concentration. Calibration curves for Ni, Pb and Ag are shown in Fig.38.

Mention should also be made of a method used by A. LACOURT[98] (1955) although it has now been superseded by her own spectrophotometric method described on page 46. Vanadium was separated from other metals by chromatography and was estimated on paper as the oxinate using a simple photometer employing a filter. The spot was then scanned mm. by mm. The most important factor lowering the accuracy of the estimation was the non-homogeneity of the paper. This was overcome by measuring the optical density of the blank paper before elution as well as the optical density of the chromatographed spots. The variations in the optical density of the blank were taken into account and enabled the true optical density of the chromatographed spot to be obtained. The logarithms of the corrected galvanometer readings were then plotted against distance measured along the strip, to give the usual distribution curve. This method did away with the uncertainties of drawing the best apparent base line, but the calculations were rather laborious.

(e) Total colour of spot - scanning the spot by a small square aperture, in two dimensions

R. J. WIEME[119] (1958) described an apparatus which permitted scanning by a spot of light measuring 2 x 2 mm. This was achieved by rotating a slit of 2 mm. in front of a slightly curved stationary slit of 25 x 2 mm. The translucent chromatogram was moved 2 mm. at a time. The output from the photo-cell was applied to a photo-multiplier circuit which had a logarithmic response. Thus the logarithm of the amount of light that had been absorbed

was integrated, and not the amount itself. The slit was rotated so fast that the galvanometer needle did not vibrate. Assuming Beer's law to hold, the galvanometer deflection would be linearly related to the quantity of substance in a given stationary slit. This enabled a curve of true optical density versus distance to be plotted, instead of apparent optical density versus distance.

The apparatus was tested by measuring several spots of amino acids of various shapes, but of the same content. The readings were found to be very much closer together than with an ordinary scanning densiometer. This method was not applied to metals, but it has obvious applications for those cases in which regularly shaped spots cannot be obtained.

It should be noted that the errors introduced in ordinary scanning of a circular spot, owing to the non-uniformity of the material over the whole area, may be lessened somewhat by reducing the slit length so that rather less than the whole width of the spot is scanned, as suggested by H. CAMPBELL[120] (1953). Alternatively, the optical system may be arranged to give brighter illumination at the middle of the slit than at the ends as in certain commercial densiometers.

(f) Continuous estimation

Brief mention should be made of a method developed by R. H. MÜLLER[121] (1949) which may be regarded as a type of scanning. A mixture of pigments was separated on a special paper chromatogram in which the spots passed through a narrow channel on the filter paper. The process of elution was followed continuously by means of a beam of light which was passed through the narrow channel and was applied to a photomultiplier tube. The method can be applied only to a substance which absorbs light and has therefore obvious limitations in inorganic work.

(g) Concluding remarks

Direct photometry on paper presents certain advantages and disadvantages when compared with other methods of estimation. Methods involving the removal of the substance from the paper are capable of high accuracy but suffer sometimes from difficulties due to incomplete removal. Furthermore, they are generally less rapid than direct photometric methods. Visual methods of estimation are usually only semi-quantitative. Other non-photometric methods such as radioactivity techniques provide valuable methods of assay in special cases but are less general in application and are usually less convenient.

Direct photometry possesses several advantages over all these methods. It is general in application, rapid, and under suitable conditions gives accurate results. It suffers however, from the disadvantage that the results may be affected markedly by slight variations in procedure. Furthermore, it is often difficult to choose the most suitable direct photometric method from a multiplicity of modifications. The general view that transmission is superior to reflection requires further investigation, as does also the practice of rendering the paper transparent. Further evidence is also required to decide between the relative merits of single reading and scanning methods of estimation.

C. EXPERIMENTAL WORK

PRELIMINARY

PHOTOMETRY OF NON-CHROMATOGRAPHED SPOTS

CHROMATOGRAPHIC SEPARATION

PHOTOMETRY OF CHROMATOGRAPHED SPOTS

§ 1 - 2

PRELIMINARY

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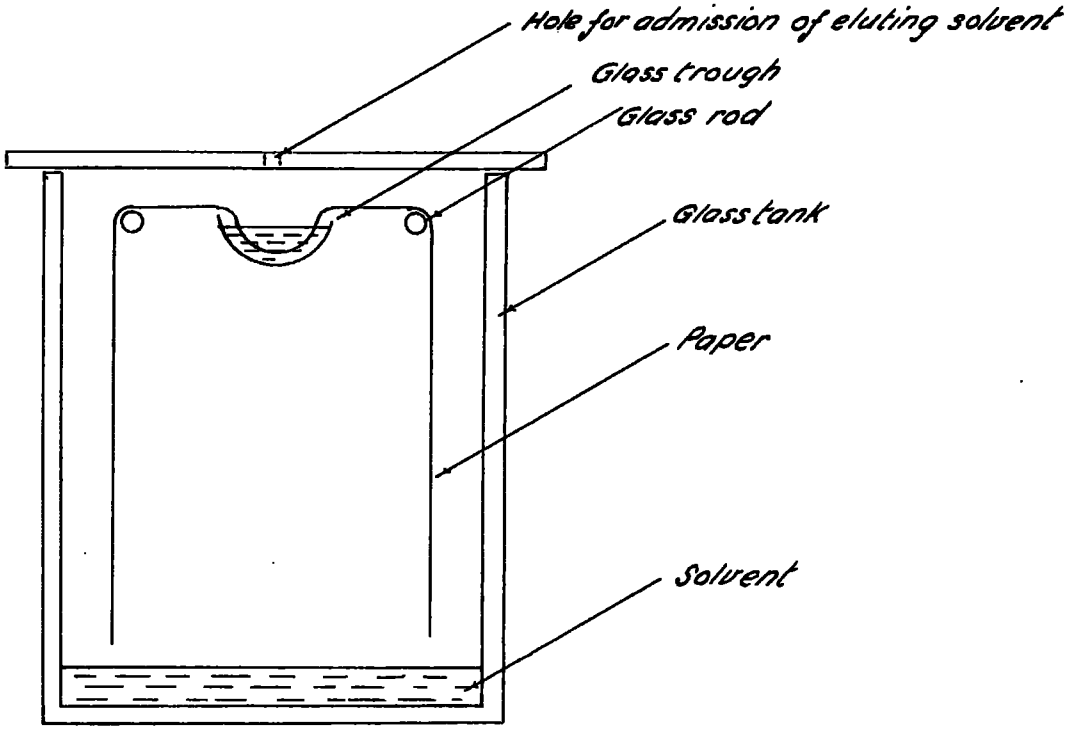

Fig. 1. Apparatus used for descending chromatography

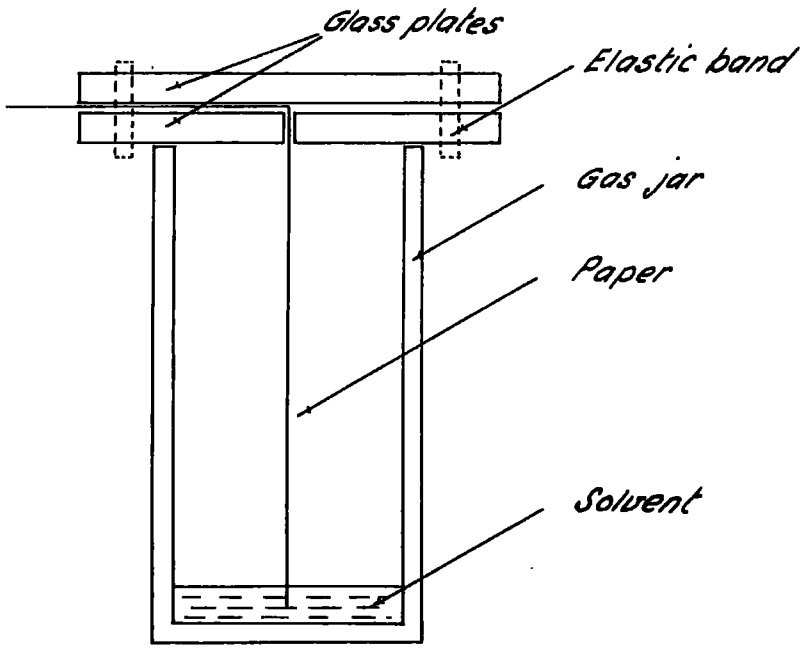

Fig. 2. Apparatus used for ascending chromatography

§ 1

AIMS AND LIMITATIONS OF EXPERIMENTAL WORK

(a) Aims of experimental work

The experimental work was essentially comparative, and was carried out with a view to studying:-

(i) Some of the factors influencing the production of chromatograms suitable for quantitative estimation. Both broad sheets and narrow strip chromatograms were investigated.

(ii) Several photometric methods of estimating metals directly on paper. In particular, an attempt was made to find the relative merits of transmission and reflection, and also of single reading and scanning methods of analysis.

(b) Limitations of experimental work

Copper and nickel were chosen for investigation for three reasons:-

(i) These metals can be readily developed on paper by rubeanic acid to give coloured complexes which do not fade in the absence of light. This simplifies comparative work, where it is necessary to estimate the same spot by different methods.

(ii) The metals in question are not present in the ordinary grades of paper, (as may be the case with calcium, for example) so careful and lengthy washing is not required[26,63].

(iii) Several methods of estimating these metals have already been described, and it is thus possible to make a direct comparison between the experimental work described here and that of other workers.

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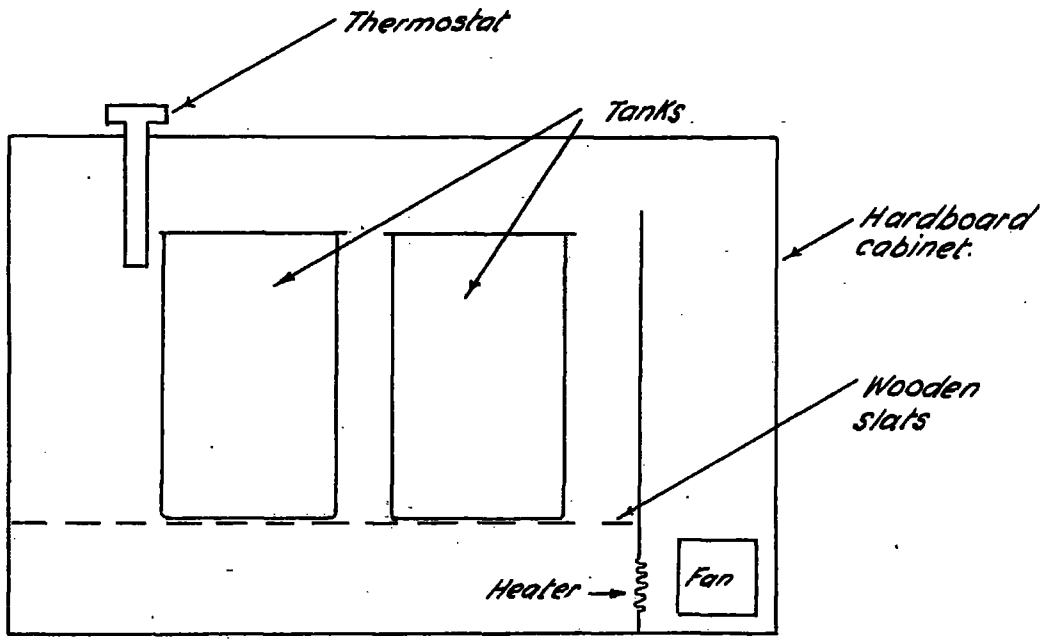

Fig. 3. Constant temperature cabinet

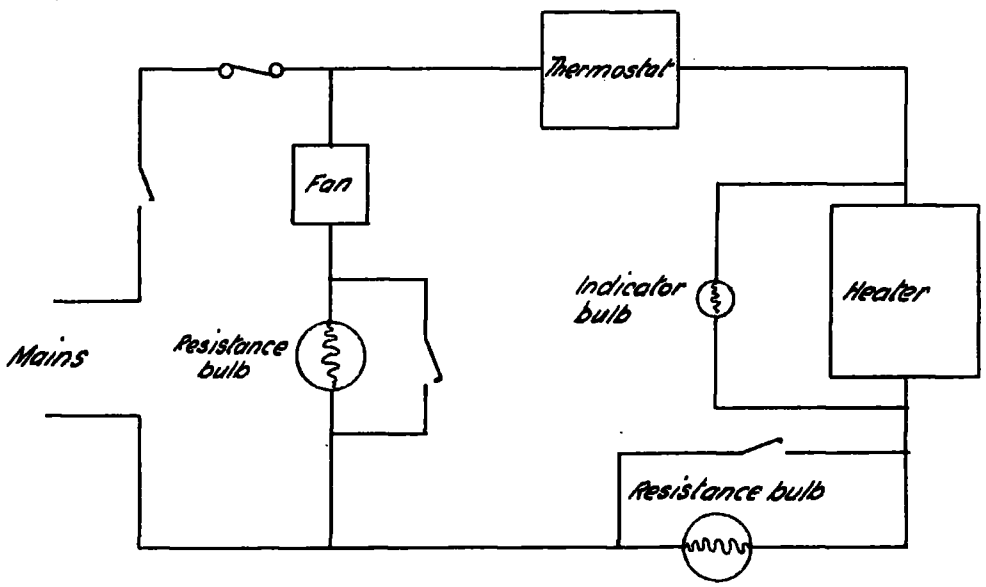

Fig. 4. Electric wiring of constant temperature cabinet

§ 2

DESCRIPTION OF APPARATUS

(a) Apparatus used for chromatography

The apparatus used for descending chromatography is shown in Fig.1. A hole in the lid allowed the eluting solvent to be added after the paper had hung in the solvent vapour for some time.

The most convenient apparatus for ascending chromatography was found to be that shown in Fig.2. It has the great advantage over other methods that the atmosphere in the gas jar can, if ncessary, be saturated before inserting the paper in the solvent vapour. Alternatively, the paper may be allowed to equilibrate in the saturated atmosphere, by hanging it so that it does not quite touch the liquid.

A constant temperature cabinet was used to ensure accurate control of temperature. This was big enough to possess a reasonably large heat capacity which is necessary for accurate thermostating. Capacity 50 cu.ft. The cabinet is shown in Figs.3 and 4. It enabled temperatures up to 15°C above room temperature to be used, but normally temperatures of about 23°C were employed. Providing the room temperature did not greatly vary, the temperature could be controlled to within $\pm$ 0·1°C.

The Agla micro-burette was used in a few experiments in which it was necessary to alter the spot volume.

Most of the work was carried out using a micro-pipette constructed from a piece of capillary tubing as shown in Fig.5. This filled immediately on touching the surface of the liquid,

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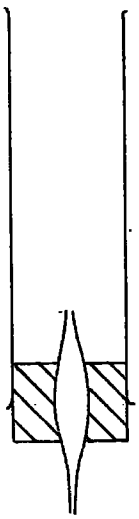

Fig. 5. Micropipette (not to scale)

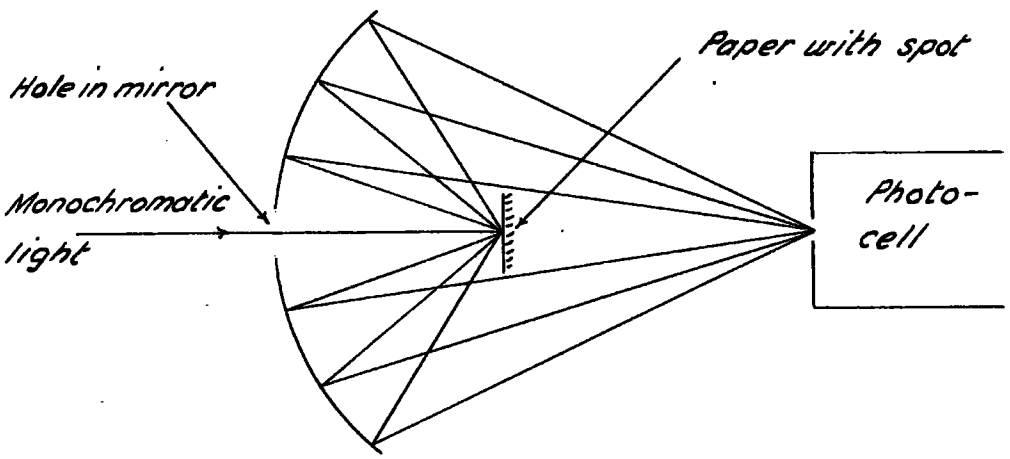

Fig. 6. Principle of reflection attachment (not to scale)

with the aid of slight suction by mouth. The contents were removed almost at once on touching the paper. Slight pressure was required to start delivery. The volume was found by filling with 1·00 N sodium hydroxide solution which was pipetted into a micro-flask containing two drops of diluted screened methyl orange. 0·100 N sulphuric acid was then run in from an Agla micro-burette. The mean of eight readings gave the value 2·64 μl(± 2%).

Whatman paper No.2 was used in all work except where the contrary is stated.

(b) Apparatus used for colour measurement

The Hilger Uvispek Model 700 Spectrophotometer was used throughout. This provides a beam of light which measures approximately 3 x 15 mm. in cross-section at the centre of the measuring compartment. The Uvispek reflection attachment was used for reflection measurements together with the appropriate supplementary lens. This lens converts the slit of light to a beam measuring approximately 2 x 2 mm. in cross-section. The light reflected from the paper is focused by a concave mirror on to the photo-cell as shown in Fig.6. The attachment contains a device which enables both the blank paper and the spot to be measured in rapid succession. The limitations of the attachment are that it does not permit scanning, nor does it allow adjustment of the paper while a light measurement is being taken.

A versatile transmission adaptor was constructed which enabled small sheets of paper to be scanned by a slit, or by a small square aperture. The adaptor could hold either dry paper, or paper immersed in a cell containing a liquid. A diagram of the adaptor is shown in Fig.7 and a detailed description is given on page 57.

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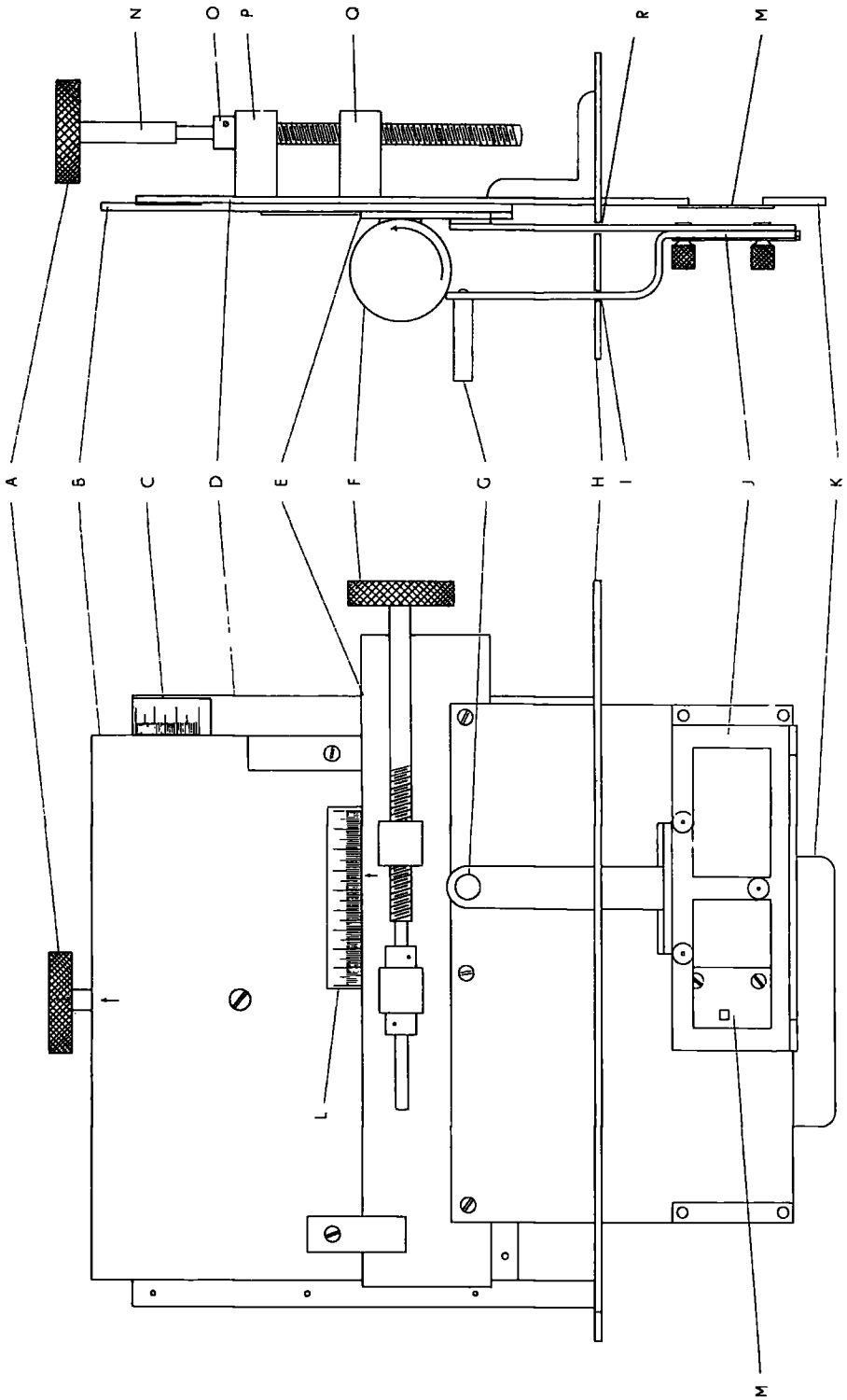

Fig.7. Scanning transmission adaptor

Description of Scanning Transmission Adaptor

The attachment is built about a horizontal plate H which fits accurately into the top of the measurement well of the spectrophotometer. To this plate is fixed rigidly a vertical plate D which extends both above and below the horizontal plate H. A plate B is free to slide up and down; being controlled by the knob A. A number 0 B.A. thread is used, one turn of which moves the plate exactly 1 mm. The collar O is fixed rigidly to the axle N. A small block of metal Q is tapped to take the thread and is attached to plate B via a slot (not shown) in plate D. The axle fits through the block P which is attached directly to plate D. The vertical position of this is recorded against a scale C.

Another plate E is free to slide from side to side and is controlled by the knob F, its position being recorded on the scale L. This plate extends through a slit R below the plate H into the measuring compartment of the spectrophotometer.

To this moveable plate is attached a holder J to take either dry paper or specially constructed cells. This holder contains two compartments, one for the spot and the other for the standard. The holder can be moved from the standard to the measure position by the handle G attached to it by means of a strip of metal which passes through a slit I in the horizontal plage H. A small square aperture M or a slit is placed in the plate E to regulate the area of the beam of light striking the paper.

The adaptor is rendered absolutely light tight by means of black velvet (not shown). A neutral density filter of approximately the same density as paper can also be rapidly inserted in, and removed from, the light beam just behind the aperture M. This is controlled by a small lever on the upper side of the plate H. This mechanism is not shown.

§ 3 - 13

PHOTOMETRY OF NON-CHROMATOGRAPHED SPOTS

The following sections are devoted to a study of the photometric estimation of non-chromatographed spots and of allied subjects.

§ 3

APPLICATION OF SPOTS TO PAPER

Neutral solutions of nickel salts applied to the paper gave rise to ring-shaped spots. Nickel salts in 3 N HCl, however, were found to give uniform spots over a concentration range of 0·05 g./l. to 4 g./l. [S. V. VAECK[57] .] This also applied to copper salts. Alcoholic rubeanic acid containing a little ammonia was used for development.

All solutions of metals were therefore made up in 3 N HCl. Nickel ammonium sulphate and copper sulphate (of analytical reagent quality) were used to prepare the standard solutions.

Solutions of Cu^{++} and Ni^{++} of various concentrations were made up in 3 N HCl for subsequent work.

§ 4

DEVELOPMENT OF NON-CHROMATOGRAPHED SPOTS

(a) Method of application of developer

Three methods of applying the rubeanic acid developer to the paper were tried:-

(i) Spraying - A simple hand spray was used, but it was found difficult to spray the paper evenly.

(ii) Dipping the paper strip in a trough in a see-saw fashion - Again this method gave irregularities. Differences in colour density between spots of the same concentration on different sheets of paper could easily be detected by eye.

(iii) Total immersion - This appeared to be the most reliable method. Spots of the same concentration on the same sheet of paper gave closely the same reflectance, although it was difficult to obtain the same value on different sheets of paper. The sheets appeared badly stained, but it was found possible to prevent this by rinsing the paper in alcohol before drying.

Total immersion was therefore used in all subsequent work.

(b) Drying the paper

Several methods of drying the paper were investigated:-

(i) Allowing the paper to 'drip-dry' in the constant temperature cabinet at 20°C immediately after development with rubeanic acid. This formed a dark brown stain all over the paper - darker at the bottom.

(ii) Blotting between sheets of clean filter paper - This prevented much brown stain remaining, but the paper appeared to possess a different surface after blotting.

(iii) Agitating the paper for half a minute in alcohol and then allowing it to 'drip-dry' in the cabinet - This was by far the most satisfactory method. No staining of the white areas could then be detected by the spectrophotometer and no running of the rubeanate appeared to take place.

Accordingly the third method of drying was chosen for future work.

(c) Method of making the spot ammoniacal

It was noticed that the colour of copper spots developed in alcoholic rubeanic acid was not by any means constant; some spots appeared yellow-green and others green. At first, this yellow-green colour was thought to be due to the action of air on the rubeanate, because spots that were developed by 'see-sawing' in the rubeanic acid were much more prone to the yellow-green colour than those developed by total immersion. (The values of reflectance for spots of the same concentration varied by as much as 10%). The following experiments were therefore carried out:-

(i) Spots were developed for half a minute in 0·05% alcoholic rubeanic acid containing 5% concentrated ammonia.

(ii) Spots were hung in ammonia vapour and then developed for half a minute in 0·05% alcoholic rubeanic acid containing no ammonia.

The results of the first method were by no means constant, the visual colour and the reflectance varying considerably. The second method gave highly reproducible results, the visual colour always being the same shade of green and the values of reflectance remaining almost constant.

(iii) Copper spots of concentration 1 g.Cu^{++}/l. (of volume 2·64 μl) applied, dried for 5 minutes, and hung in ammonia for half a minute. Some were then developed in 0·1% alcoholic rubeanic acid, while others were developed in alcoholic rubeanic acid containing 0·1% concentrated ammonia.

The spots were then washed in alcohol, dried, and measured by transmission at 660 mμ., the transmittance at the centre being noted. The results obtained are shown in Table 1; the minimum transmittance indicating the most complete development.

Table 1

Effect of ammonia in rubeanic acid on complete development of copper

Time	Values of % transmittance					
	10secs	30secs	2mins	10mins	40 mins	2hrs
Rubeanic acid	5·2	4·5	4·1	4·2	4·3	5·1
Rubeanic acid containing 0·1% concentrated ammonia	8·7	7·1	4·6	4·9	4·5	-

Clearly, the presence of ammonia in the rubeanic acid does not lead to more complete development. Development appears to be virtually complete after one or two minutes.

(iv) Three methods of applying ammonia after development were then compared:-

Method 1 Three spots of concentration 1 g.Cu^{++}/l. were placed on paper. After drying for 5 minutes they were held in ammonia vapour for half a minute, developed in 0·1% alcoholic rubeanic acid (without ammonia) for two minutes, washed in alcohol for half a minute, and dried for five minutes before measuring in the spectrophotometer.

Method 2 The same procedure was carried out on a further three spots, except that after development in rubeanic acid, the spots were immersed in 0·5% alcoholic ammonia for half a minute before washing and drying.

Method 3 The same procedure was carried out on three more spots, but this time the paper was held in ammonia vapour for half a minute after development instead of immersing in 0·5% alcoholic ammonia.

The results are shown in Table 2. (overleaf)

Table 2

Effect of modifying the method of applying ammonia to the paper

Method	Transmittance %	Mean Transmittance %
1	4·37 4·47 4·37	4·40
2	4·18 4·13 4·27	4·17
3	4·20 4·07 4·10	4·12

It thus appears that treatment with ammonia vapour after immersion in rubeanic acid leads to a more complete development. Method 3 was more convenient and perhaps slightly more effective and was chosen for subsequent work in preference to Method 2.

The following method was chosen for the development of both chromatographed and non-chromatographed spots.

Spots dried at least five minutes in constant temperature cabinet at 20°C

Hung in ammonia vapour for half a minute

Immersed in 0·1% alcoholic rubeanic acid for one minute with agitation

Hung in ammonia vapour again for half a minute

Washed in alcohol for half a minute

Dried at least five minutes in cabinet at 20°C before measuring in the spectrophotometer

Facing P. 64

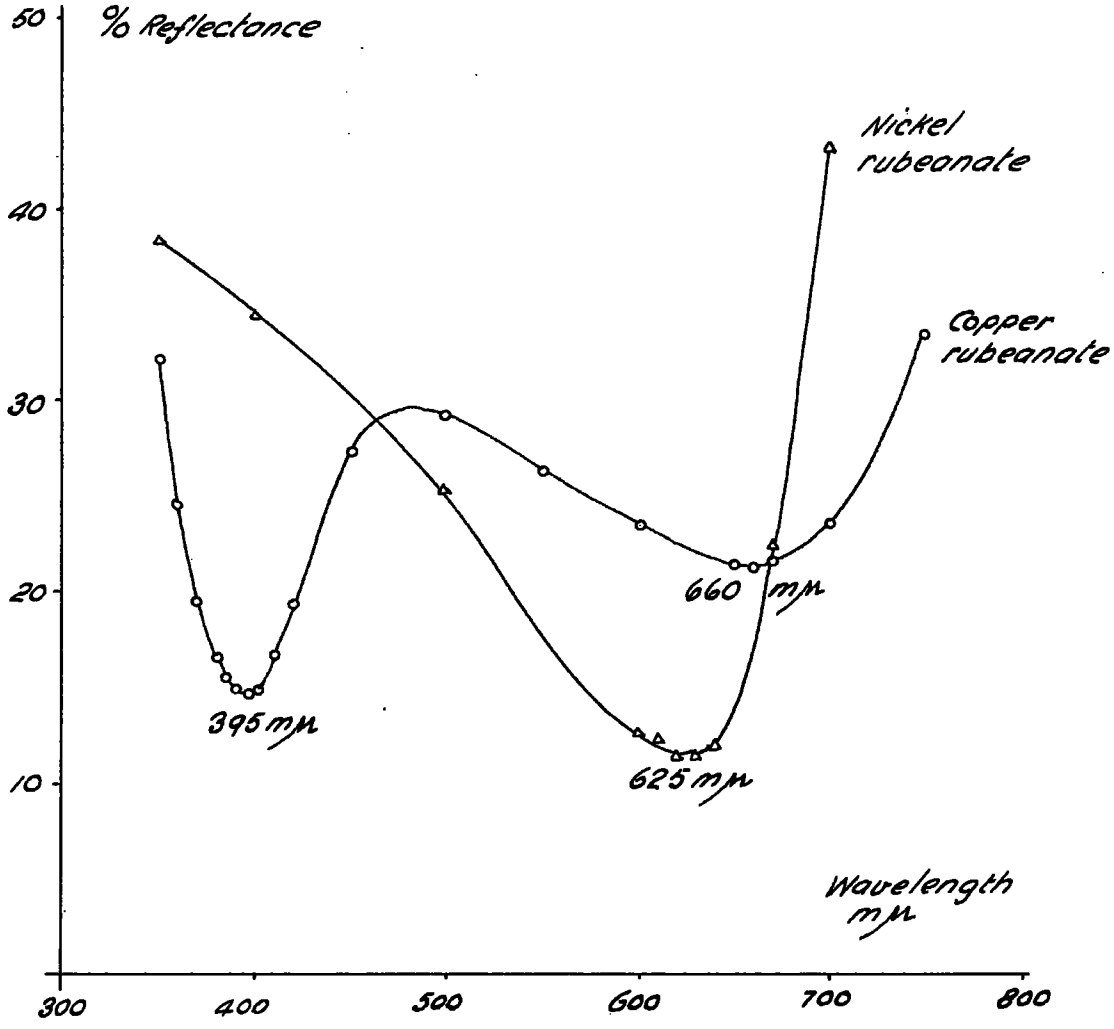

Fig. 8. Reflectance versus wavelength curves of copper and nickel rubeanates

§ 5

DETERMINATION OF ABSORPTION MAXIMA

Copper and nickel spots each of volume 2·64 µl. and concentration 1 g./l. were placed on paper. The reflectance at various wavelengths was measured, as shown in Table 3. The reflectance versus wavelength curves are shown in Fig.8.

Table 3

Reflectance of copper and nickel rubeanates at various wavelengths.

Copper		Nickel	
Wavelength mµ	% Reflectance	Wavelength mµ	% Reflectance
350	32·1	350	38·5
360	24·6	400	34·5
370	19·6	500	25·5
380	16·3	600	12·7
385	15·4	610	12·1
390	14·8	620	11·4
395	14·7	630	11·4
400	15·0	640	11·9
410	16·6	670	22·3
420	19·2	700	43·2
450	27·3	800	89·5
500	29·2		
550	26·2		
600	23·7		

Table continued overleaf/

Copper		Nickel	
Wavelength mμ	% Reflectance	Wavelength mμ	% Reflectance
650	21·4		
660	21·3		
670	21·6		
700	23·7		
750	33·2		
800	48·5		
900	80·2		

From these measurements, it is clear that the absorption maxima occur at:-

Copper rubeanate 395 mμ and 660 mμ

Nickel rubeanate 625 mμ

It was also confirmed that the absorption maxima occurred at the same wavelengths when the readings were taken by transmission.

The absorption maximum of 660 mμ was used for copper rubeanate, as the spectrophotometer was found to be more sensitive at this wavelength than at 395 mμ. The spectrophotometer did not give accurate readings at its extreme limit of sensitivity.

A slit width of 0·1 mm. giving a band of 0·5 mμ, was used for reflectance measurements. The slit width was increased to 0·3 mm. for transmittance measurements on dry paper in order to admit more light to the photo-cell.

The following spectrophotometer settings were used for all subsequent work:-

Absorption maxima used: Copper 660 mμ
: Nickel 395 mμ

Slit widths used: reflection 0·1 mm.
: transmission 0·3 mm.

§ 6

CHOICE OF BACKGROUND FOR REFLECTION MEASUREMENTS

Reflection measurements incorporate a certain degree of transmission, as some of the light passes through the paper and is reflected back to the photo-cell. The effect of using different backgrounds was therefore investigated.

(a) Use of several sheets of white paper

Nine sheets of white filter paper were placed in each compartment of the reflection attachment and the reflectance at 660 mμ was measured. The sheet of paper furthest from the reflecting surface was then removed and the reflectance of the remaining eight sheets was measured. This procedure was repeated until all the sheets had been removed. The results are shown in Table 4 and Fig.9.

Table 4 overleaf

Facing P.67.

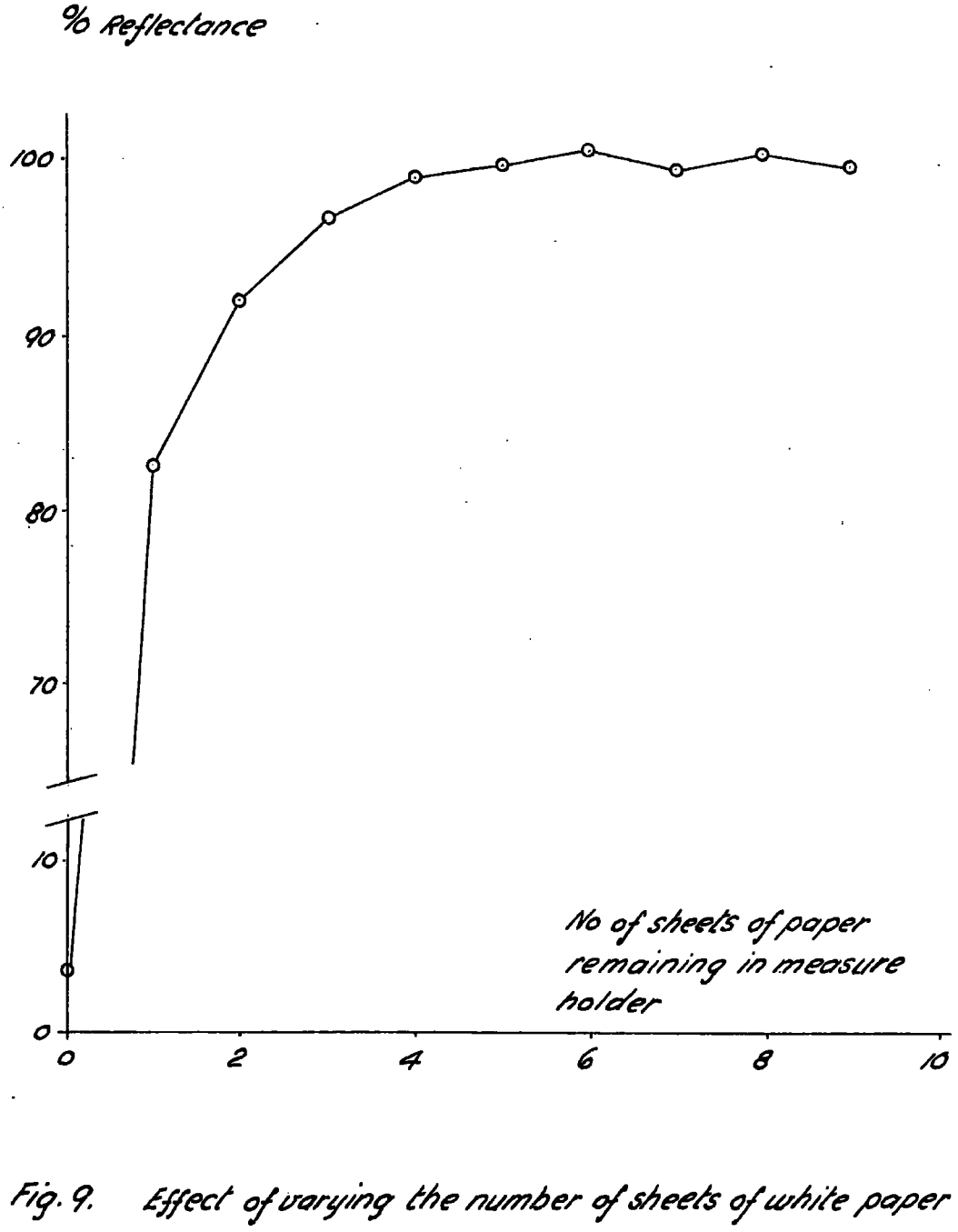

Fig. 9. Effect of varying the number of sheets of white paper used as a background (9 sheets white paper in the standard holder)

Table 4

Effect of varying the number of sheets of white paper used as a background [9 sheets of white paper in the standard holder]

No. of sheets remaining in the 'measure' compartment	% Reflectance
9	99·6
8	100·3
7	99·6
6	100·5
5	99·7
4	99·0
3	96·8
2	92·0
1	82·4
0	3·5

It thus appeared that 5 or 6 sheets of paper were necessary.

(b) Use of other backgrounds

Black velvet and also a silver mirror were tried as a background. Measurements made with black velvet appeared to be rather less reproducible than those made with white paper, while those made with a silver mirror and with white paper had about the same reproducibility.

All reflection measurements in the ensuing work were carried out with a background of six sheets of white paper, except when the contrary is stated.

§ 7

CHOICE OF STANDARDS FOR TRANSMISSION MEASUREMENTS

The choice of a suitable standard for transmission readings is considerably more difficult than for reflection readings. At first, using the scanning adaptor, the piece of paper chosen as the standard was placed in the standard window of the adaptor, and the spot was placed in the measure window. This method, which will be termed the 'direct standard method' is satisfactory, provided that the area of the slit is not too small. A certain amount of work was done, however, using very small adaptor slit widths and it was found that the direct standard method did not then give satisfactory results. For if the instrument was set to 100% transmittance on only one position of the blank, the part of the paper chosen might be rather more or less opaque than the average. This could result in increasing or decreasing all the readings on the spot by as much as 10%. Alternatively the instrument could be set to 100% transmittance each time a reading was taken. This does admittedly, lead to a certain cancelling out of errors, but it has the disadvantage of imposing various errors, some positive and some negative, on each of the readings, which makes the interpretation of the results much more difficult, particularly in work in which the uniformity of the paper is being studied.

In order to overcome this difficulty, a method which will be termed the 'indirect standard method' was employed.

The adaptor was fitted with a filter of approximately the same optical density as paper. This filter (a piece of photographic film was used) could be inserted in, or removed from, the path of the light beam by moving a small lever on the scanning adaptor. Readings were then taken on each spot:-

'standard' window - empty

'measure' window - paper with spot.

(i) Place filter in beam of light, set adaptor to 'standard', adjust the instrument to 100% transmittance

(ii) Remove filter, set adaptor to 'measure' and take transmission readings as the paper is moved across the beam of light.

The piece of paper chosen to act as a standard was then placed in the 'measure' window, the 'standard' window again being left empty. Readings were taken as described above, a series being obtained which scanned a representative area of the paper.

The mean transmittance of the standard paper was noted and this enabled each reading on the spot to be expressed as a percentage of the mean transmittance of the blank paper.

All transmission measurements in the ensuing work were carred out using the indirect standard method. This was more accurate than the direct standard method, although considerably more laborious.

§ 8

UNIFORMITY OF DRY PAPER

One of the chief factors which reduces the accuracy of photometric estimation on paper is the non-homogeneity of the paper itself. The following work was carried out in order to study the uniformity of blank paper by transmitted and by reflected light.

(a) Effect of changing area of scanning slit on the variations in transmission readings

It is clear that slits of area comparable to the area of inhomogeneity of the paper are to be avoided. The same sheet of paper was scanned every 2 mm. using an aperture of 2 x 2 mm. and also every 3 mm. using a slit of 3 x 5 mm. The results shown in Table 5 and in Fig.10 make it clear that areas much less then 15 sq. mm. should not be used.

Table 5 overleaf

Facing P. 71

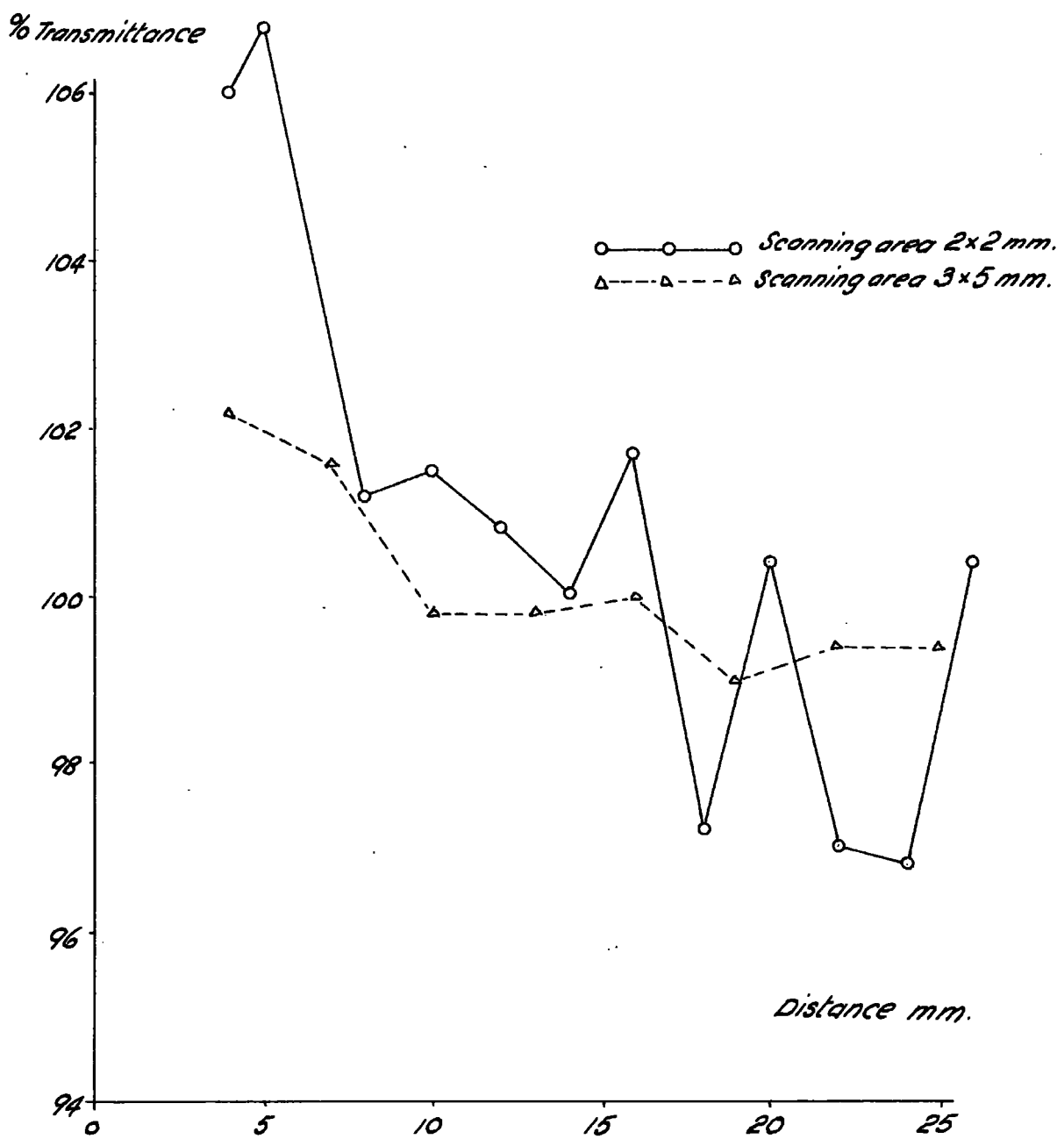

Fig.10. Effect of area of scanning slit on variation in transmittance of white paper

Table 5

Effect of area of scanning slit on variation in transmittance of white paper

Distance mm.	% Transmittance (2 x 2 mm. aperture)	% Transmittance (3 x 5 mm. slit)
4	106·0	102·2
5		
6	106·8	
7		101·6
8	101·2	
9		
10	101·5	99·8
11		
12	100·8	
13		99·8
14	100·0	
15		
16	101·7	100·0
17		
18	97·2	
19		99·0
20	100·4	
21		
22	97·0	99·4
23		
24	96·8	
25		99·4
26	100·4	

An aperture of 1 x 1 mm. was also tested, but the amount of light reaching the photo-cell was so small that accurate readings were difficult. Large slits were found to reduce further the variations in transmittance.

Since only a few cm. of paper had been scanned, it seemed desirable to test widely different points on the same reel of paper. This was done by taking samples of paper, at approximately 25 cm. intervals, along a reel. The transmittance readings obtained, using a slit of 3 x 5 mm. are shown in Table 6.

Table 6

Variation in transmittance of white paper at widely different points (slit area 3 x 5 mm.)

Sample No.	1	2	3	4	5	6
% Transmittance	100·0	97·5	98·5	100·6	100·1	101·3

The variation is only slightly greater here than that shown in Table 5.

(b) Uniformity of paper by reflected light

Twelve circles were cut out of a sheet of filter paper and their reflectance was measured. The results are shown in Table 7.

Table 7 overleaf

Table 7

Variations in reflectance of white paper (area 2 x 2 mm.)

Sample No.	1	2	3	4	5	6
% Transmittance	99·3	100·6	100·4	100·0	99·8	99·8
Sample No.	7	8	9	10	11	12
% Transmittance	100·2	99·5	100·0	100·0	100·5	99·8

(c) Comparison of uniformity of blank paper by transmission and reflection

The readings taken by transmission with an aperture of 2 x 2 mm. recorded in Table 5, may be compared with those obtained by reflection in Table 7, as shown in Table 8.

Table 8

Comparison of uniformity of white paper by transmission and reflection (area 2 x 2 mm. in both cases)

Standard deviation of 12 transmission readings	± 3·7%
Standard deviation of 12 reflection readings	± 0·4%

Summary

1. Small scanning areas (less than 15 sq. mm.) are to be avoided in transmission measurement.

2. White paper appears much more uniform by reflected than by transmitted light.

§ 9

UNIFORMITY OF PAPER IMMERSED IN LIQUIDS

It has been claimed[79,83] that immersion of paper in a liquid of approximately the same refractive index increases its optical uniformity. S. V. VAECK[74] reported, however, that immersion of paper in a liquid reduces its uniformity. The following work investigates the effect of several liquids, and of different methods of application to the paper.

Two methods were used for rendering the paper transparent:-

(i) The paper was placed in a cell with the liquid. The construction of the cell is shown in Fig.11.

(ii) The paper was dipped in a liquid and then allowed to drain for some time. It was then measured directly, without glass on either side.

The first of these methods was used with several liquids:-

(i) Xylene ($\mu = 1{\cdot}49$)

The paper was allowed to soak in a beaker of xylene for a few minutes and was then transferred to the cell which had been previously filled with xylene. The excess xylene was wiped off and the cell was placed in the scanning adaptor. It rapidly became clear that there were two difficulties. First, the readings varied with time, and secondly, the cell had to be refilled from time to time, on account of evaporation.

A piece of paper was scanned dry. It was then immersed in xylene and scanned after 10, 15, 30, and 60 minutes. A small aperture of 2 x 2 mm. was used, in order to show up the variations. [See Table 9 and Fig.12]

Facing P.75

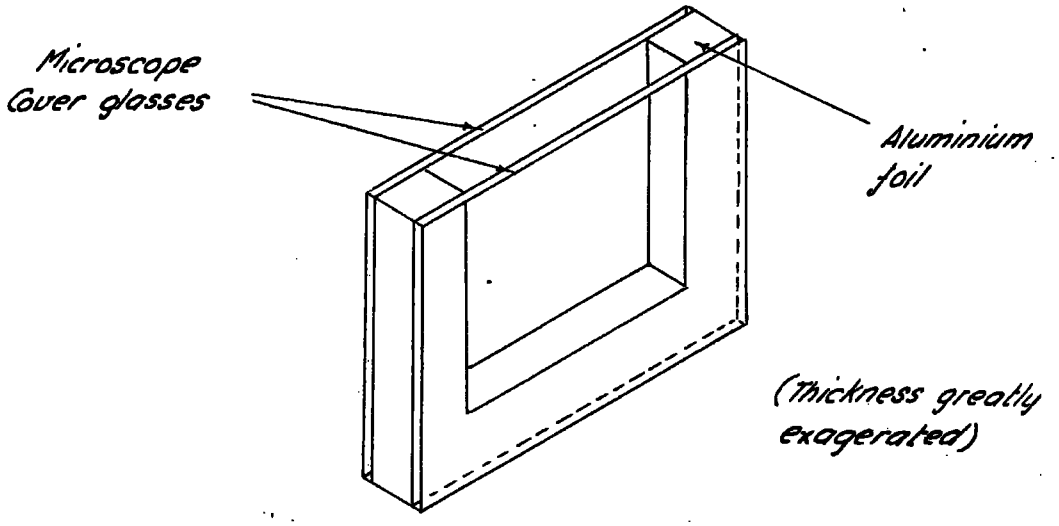

Fig. 11. Cell used for holding paper in liquids

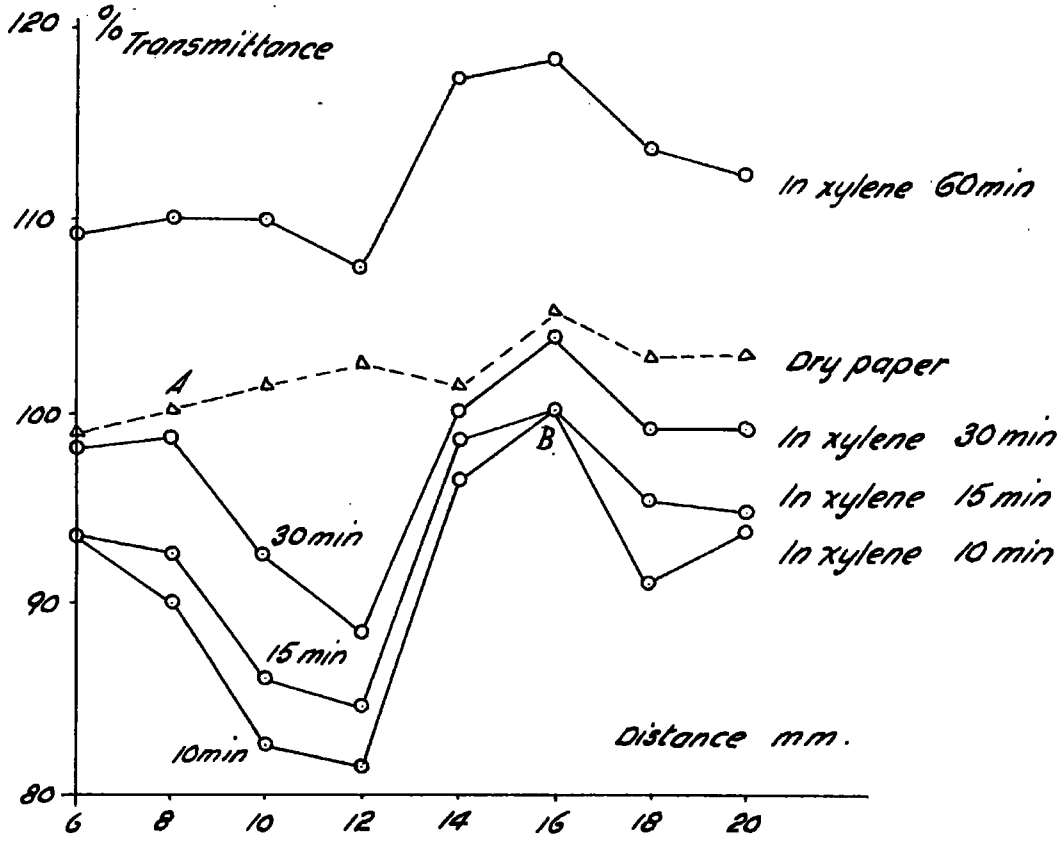

Fig. 12. Variations in transmittance of paper dry, and immersed in xylene.

Table 9

Variations in transmittance of paper dry, and immersed in xylene

Distance mm.	% Transmittance [relative to reading A as 100] dry	% Transmittance in xylene [relative to reading B as 100] after: 10min.	15min.	30 min.	60min.
6	98·8	93·5	93·5	98·0	109·3
8	100·0(A)	90·0	92·6	98·6	110·0
10	101·4	82·5	86·0	92·6	110·0
12	102·5	81·5	84·6	88·3	107·3
14	101·4	96·2	98·3	100.0(B)	117·4
16	105·2	100·0	100·0	104·0	118·3
18	103·0	91·0	95·2	99·0	113·7
20	103·0	93·8	94·8	99·0	112·5

Immersion of the paper in xylene greatly increased its transparency, but appeared to decrease rather than increase its optical uniformity.

(ii) Water

The same procedure was carried out, exccpt that water was used. [See Table 10 and Fig.13]

Table 10 overleaf

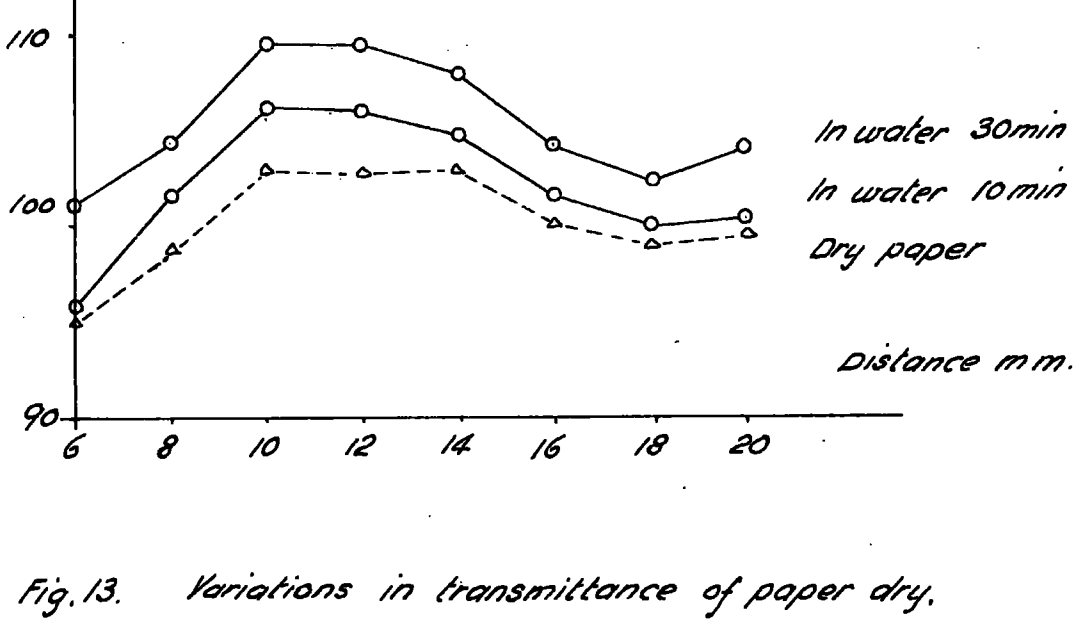

Fig. 13. Variations in transmittance of paper dry, and immersed in water

Table 10

Variations in transmittance of paper dry, and immersed in water

Distance mm.	% Transmittance dry	% Transmittance in water after 10 min.	30 min.
6	95·0	95·9	101·0
8	98·9	101·5	104·4
10	103·0	106·2	109·5
12	102·8	106·0	109·5
14	102·8	104·8	108·0
16	100·0	101·5	104·2
18	99·0	100·0	102·2
20	99·3	100·2	104·0

Water did not render the paper as transparent as xylene, since its refractive index is considerably less than that of paper fibres, but the transmittance did not change much after 30 minutes. The optical uniformity of paper immersed in water, appeared to be about the same as that of dry paper.

(iii) Paraffin, Paraffin diluted with petrol ether, glycerine, and glycerine diluted with water.

Each of these liquids was investigated. In no case was there any observable improvement in the uniformity of the paper.

(iv) Paraffin

The same procedure as above was carried out, except that after immersion in paraffin, the paper was subjected to a vacuum in a desiccator for five minutes in an attempt to remove microscopic air bubbles which might have been responsible for increasing the lack of uniformity of the

paper. This procedure, used by A. L. LATNER[81], did not lead to any noticeable improvement in the uniformity of the paper.

The second method of rendering the paper transparent, that of dipping the paper in a liquid and then allowing the paper to drain, was then investigated. The paper was immersed in paraffin for five minutes, allowed to hang in the air for one hour, and lightly blotted. This method, which has been described by R. J. BLOCK[87], appeared to be the most satisfactory of the methods investigated. The readings did not vary with time although they did vary to a slight extent with the amount of paraffin remaining on the paper.

Conclusions

(i) In none of the cases studied was there any improvement in the optical uniformity of the paper to transmitted light. It should be noted the work was carried out in a spectrophotometer and that the photo-cell subtended only a small angle at the paper.

(ii) The most convenient method of rendering the paper transparent was that of immersion in paraffin oil, followed by hanging in the air for an hour.

(iii) Total immersion of the paper in a cell containing a liquid is, generally, an unsuitable method, as the transmittance varies with time for as long as an hour.

Most subsequent work was carried out using dry paper. Some work was, however, carried out on transparent paper, and the method chosen was that of immersion in paraffin, followed by hanging in the air for one hour.

§ 10

EFFECT OF TIME OF DRYING AND HUMIDITY OF AIR ON TRANSMITTANCE OF DRY PAPER

(a) Effect of variation of drying time between development of spot and measurement

After developing the spots in rubeanic acid, the paper was washed in alcohol before drying. Since it is not possible in practice to measure all the spots simultaneously, the effect of drying time on transmittance was investigated.

A sheet of paper was cut to fit the scanning adaptor and then immersed in alcohol. It was allowed to dry in the cabinet. After 4 minutes, it appeared dry to the eye. After 5 minutes, the transmittance of one part of the paper was measured. The paper was then returned to the cabinet for a further 15 minutes before reading the transmittance of the same part of the paper again. The two readings were identical. This suggested that the paper is fully dried after 5 minutes.

(b) Effect of humidity variations on change in transmittance of paper

The humidity of the laboratory in winter was found, on occasion, to be as low as 40% (using Regnault's method). A variation of 40 to 80% relative humidity therefore seemed to be quite possible. For this reason the effect of humidity changes on the transmittance of the blank paper and of non-chromatographed spots, was investigated.

Accordingly, two spots, each of volume 2·64 μl. and of concentration 0·4 g.Cu^{++}/l. and 0·1 g. Cu^{++}/l. respectively, were developed and cut to fit the adaptor, so that they could be rapidly placed in it after humidification. A sheet of blank paper was also cut in the same way. These sheets of paper were then hung for a week in gas jars containing saturated solutions of potassium bromide at 20°C., in order to humidify them at 84% relative humidity. Each paper was then taken out in turn, placed in the adaptor quickly, and the reading taken at once on a day in which the humidity of the laboratory was 45%. The reading fell slowly for about half an hour and the remained constant a few % below the original value. The readings are shown in Table 11.

Table 11

Effect of humidity on transmittance of white paper and copper spots

Concentration g.Cu^{++}/l. Spot volume 2·64 μl.	Initial % Transmittance T_1	Final % Transmittance T_2	% Fall $\frac{100(T_1-T_2)}{T_1}$
0·4	15·1	14·6	3·3
0·1	49·0	48·0	2·0
0	102·1	100·0	2·1

In practice, it is not the % fall in transmittance of the spot which matters but the ratio (fall in transmittance of spot)/(fall in transmittance of white paper). These readings suggest that this ratio is not greatly affected by the humidity at which the readings are taken.

In subsequent work, the paper, wet with alcohol, was

always allowed to dry in the cabinet for at least five minutes before measuring. No attempt was made to control the humidity of the paper while transmission readings were taken.

§ 11

CALIBRATION CURVES FOR NON-CHROMATOGRAPHED SPOTS BY REFLECTION

[The relation between the reflectance and the concentration of non-chromatographed spots was considered on pages 34 and 35. In the following work, the ratio of absorption coefficient to scattering coefficient is denoted by K/S. This ratio may be obtained from the reflectance using the Table given in Appendix 2.]

(a) Calibration curves using dry paper

Copper and nickel spots of various concentrations were placed on the same sheet of paper, developed, and measured by reflection. The results are shown in Table 12 and Figs.14 and 15. It is seen that, on plotting K/S versus concentration, the points lie close to a straight line, over a fairly wide concentration range.

Table 12 overleaf.

Facing P. 82

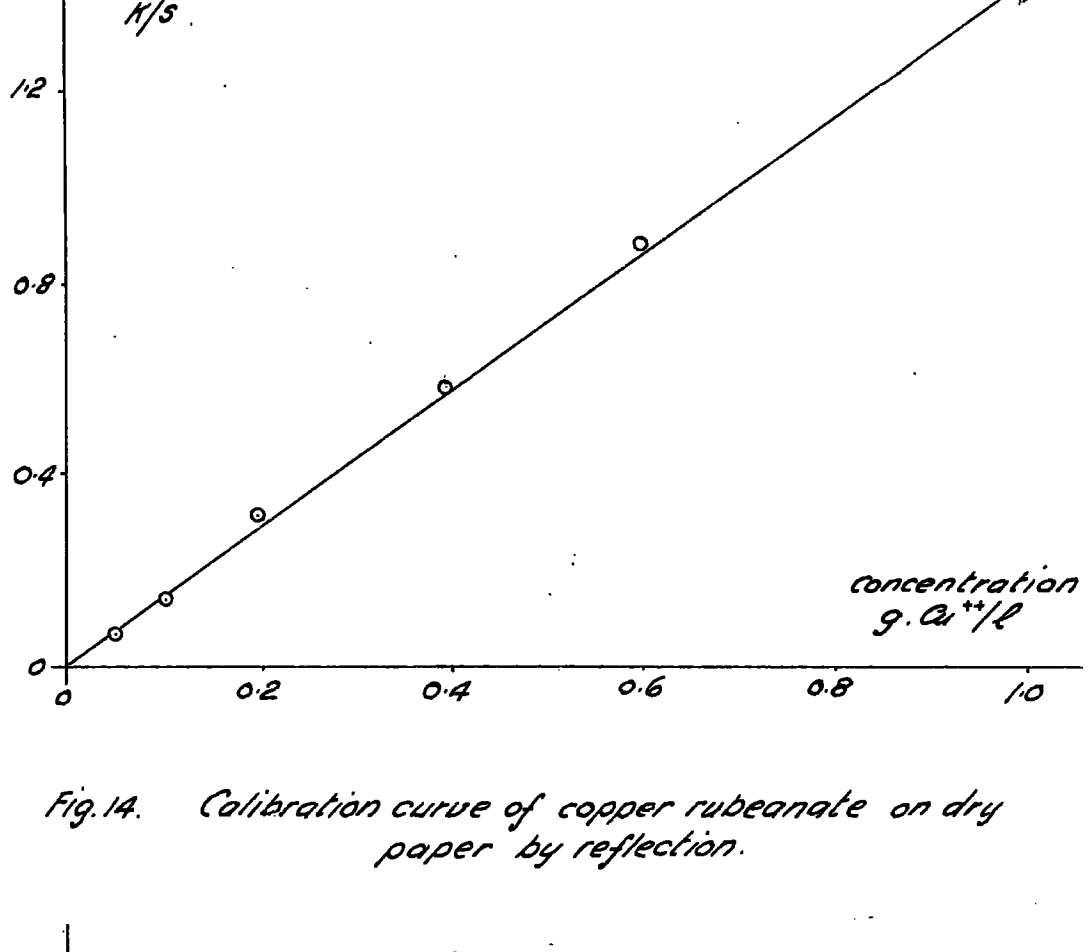

Fig. 14. Calibration curve of copper rubeanate on dry paper by reflection.

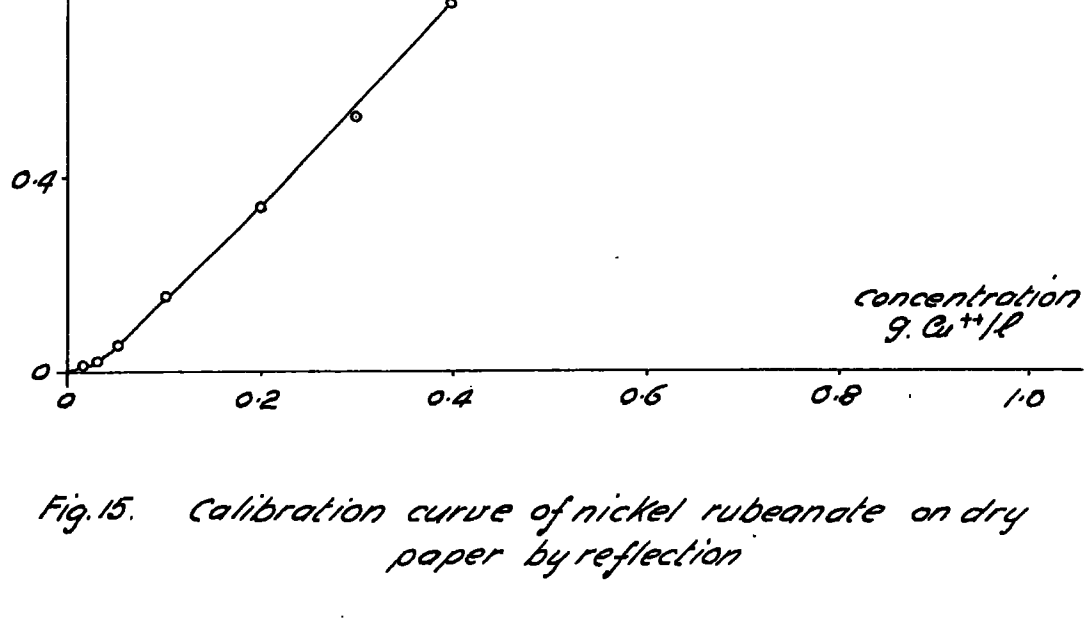

Fig. 15. Calibration curve of nickel rubeanate on dry paper by reflection

Table 12

Reflectance of copper and nickel rubeanate on dry paper

Concentration g.Cu^{++}/l.	Copper % Reflectance	K/S	Concentration g.Ni^{++}/l.	Nickel % Reflectance [Mean of 3 readings]	K/S
0·05	68·9	0·07	0·0125	88·1	0·008
0·1	59·2	0·14	0·025	82·7	0·018
0·2	46·4	0·31	0·05	75·4	0·040
0·4	35·6	0·58	0·10	58·2	0·15
0·6	28·8	0·88	0·20	44·8	0·34
1·0	21·8	1·40	0·30	37·2	0·53
			0·40	31·2	0·76

(b) Calibration curves using transparent paper by reflection

It should be understood that so-called reflectance measurements even on dry paper incorporate a considerable degree of transmission. Some of the light passes through the paper, is reflected by the white paper background, and passes back through the paper again to be measured. Any method which increases this degree of transmission might well lead to more reproducible results, if the colour is unevenly distributed at different depths within the paper. Accordingly, a modification of the reflection method was attempted to see whether any improvement was noticeable.

The paper was backed by a layer of magnesium carbonate over which a cover slip was laid. A drop of paraffin was placed on this, and the white paper or spot was then placed thereon. A further drop of paraffin was added and another cover slip placed on top. Both the standard and the spot

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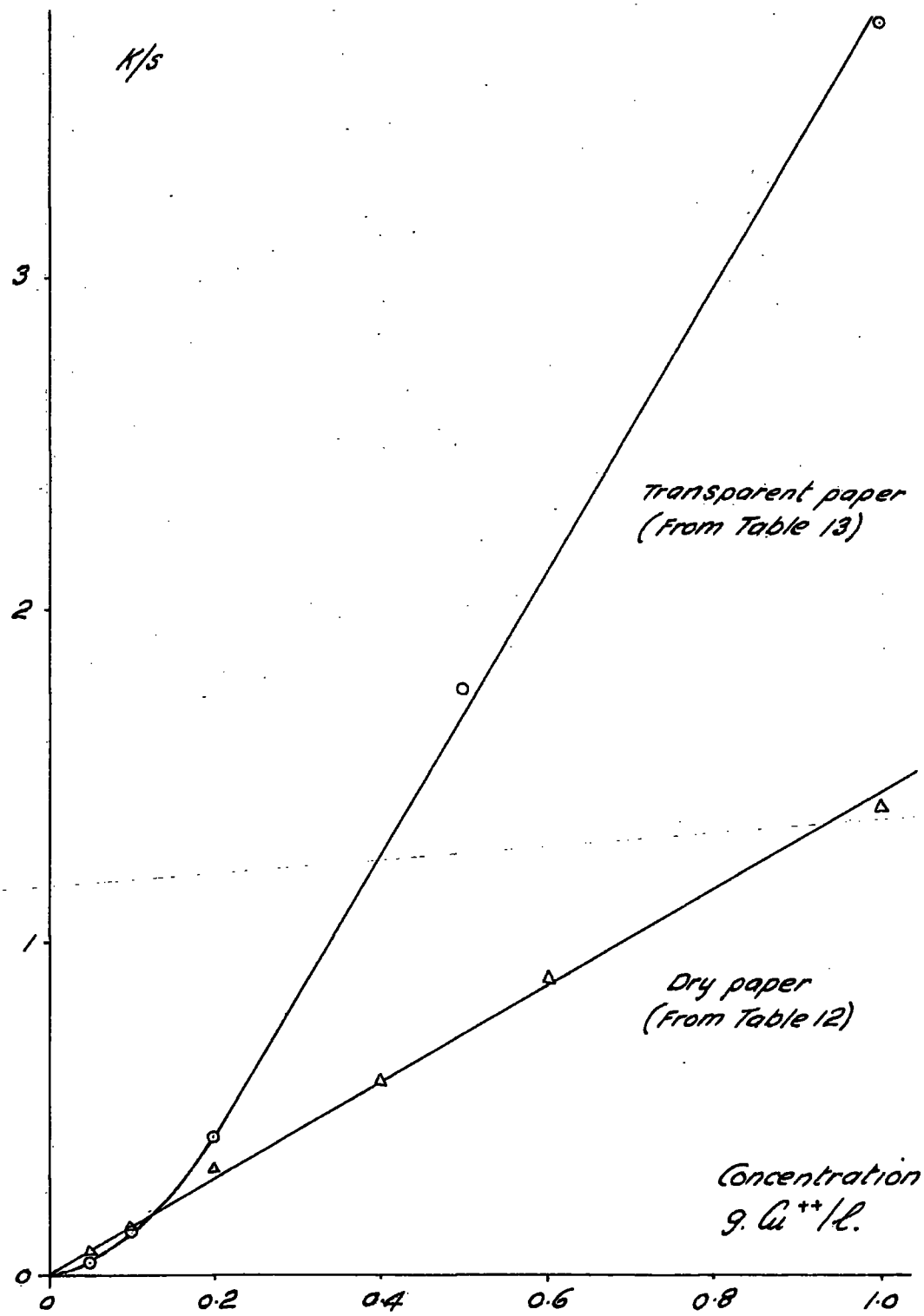

Fig. 16. Calibration curves of copper rubeanate on dry and on transparent paper by reflection.

were treated in the same manner and the reflection reading was taken in the usual way. The readings obtained are shown in Table 13 and Fig.16.

Table 13

Reflectance of copper rubeante on transparent paper, with white background

Concentration g.Cu^{++}/ℓ	% Reflectance	K/S
0·05	74·5	0·04
0·1	60·7	0·13
0·2	41·8	0·41
0·5	18·8	1·75
1·0	10·6	3·77

Unfortunately, the method suffered from all the difficulties inherent in rendering paper transparent; the reading varied with time quite rapidly, the quantity of paraffin used affected the reading, and no doubt errors might be caused by entrapped air. With considerable care, the method gave readings which lay on a smooth curve on the K/S versus concentration plot. The method, however, took a long time and required considerable attention to detail.

(c) Comparison of suitability of using dry with transparent paper for reflection measurements

The calibration curve obtained using transparent paper is steeper and would be expected to give more accurate results, if the transmittance readings are equally reproducible to those obtained with dry paper. Against this,

must be set the practical difficulties of the method and the great increase in total time required for a reflection measurement, when the paper is rendered transparent.

For routine procedure, reflection measurements on dry paper were more suitable than those on transparent paper and were used in all subsequent work.

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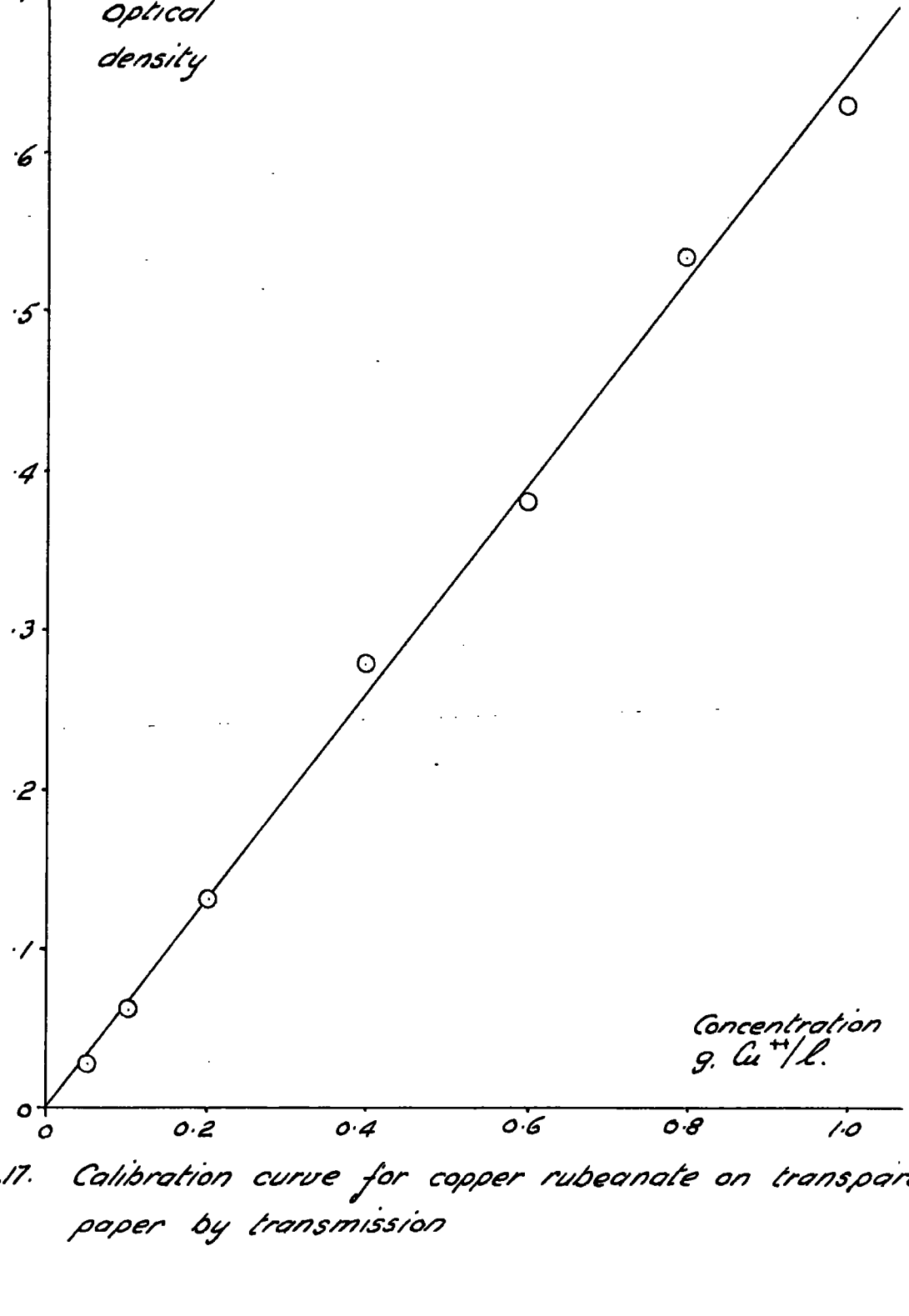

Fig.17. Calibration curve for copper rubeanate on transparent paper by transmission

§ 12

CALIBRATION CURVES FOR NON-CHROMATOGRAPHED SPOTS BY TRANSMISSION

(a) Calibration curves using transparent paper

Copper spots of various concentrations were placed on the same sheet of paper, developed, and dried. The paper was then rendered transparent by dipping in paraffin oil and allowing to hang for one hour in the air. The optical density of each of the spots was measured. The readings are shown in Table 14, and in Fig.17.

Table 14

Optical density of copper rubeanate by transmission on transparent paper

Concentration g.Cu^{++}/l.	Optical density
0·05	0·028
0·1	0·060
0·2	0·131
0·4	0·280
0·6	0·380
0·8	0·535
1·0	0·630

The calibration curve of optical density versus concentration is a straight line over the concentration range studied.

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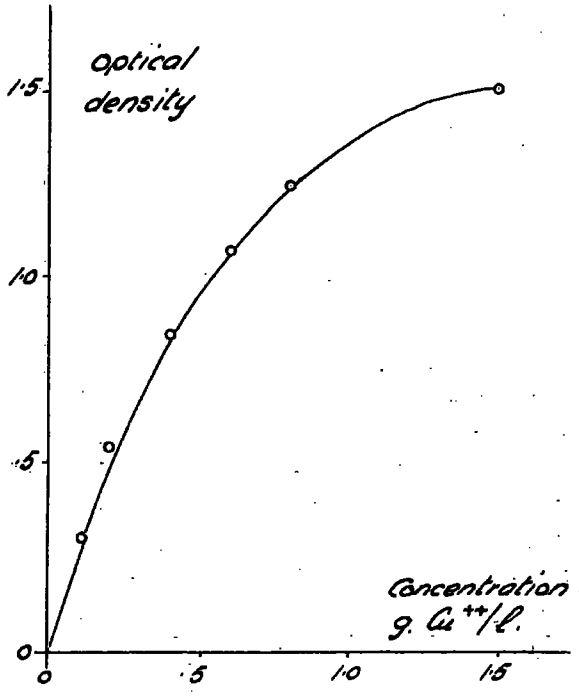

Fig. 18a

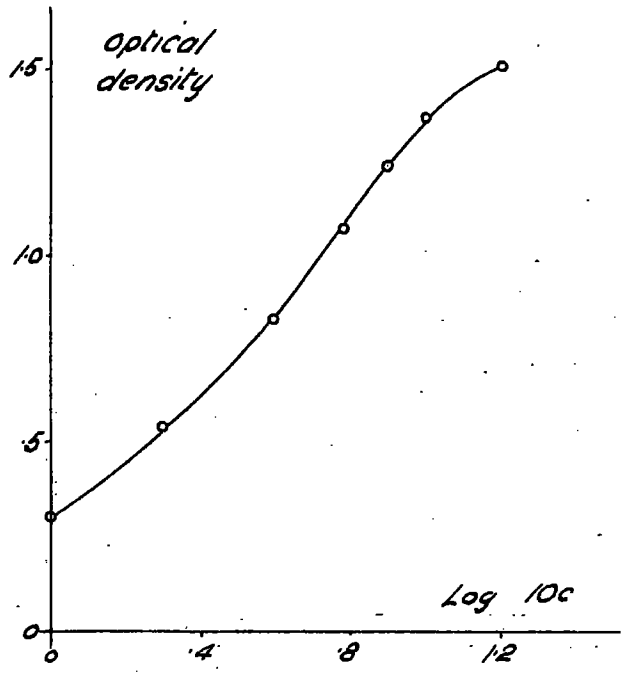

Fig. 18b

Figs. 18a, b and c.

Calibration curves for copper rubeanate on dry paper by transmission

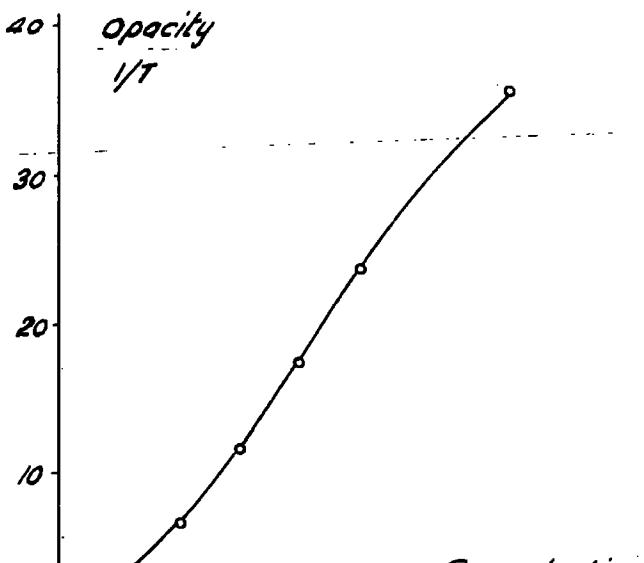

Fig. 18c (See Fig. 19 for large scale graph)

(b) Calibration curves using dry paper

The same procedure was carried out, but using dry paper. The readings are shown in Table 15.

Table 15

Functions of transmittance and concentration of copper rubeanate on dry paper

Concentration c g.Cu^{++}/l.	$Log_{10}10c$	Transmittance T [Mean of 3 readings]	Optical Density $Log_{10}\frac{1}{T}$	Opacity $\frac{1}{T}$
0·1	0.000	·506	·30	1·98
0·2	0·301	·289	·54	3·46
0·4	0·602	·146	·84	6·85
0·6	0·778	·0858	1·07	11·65
0·8	0·903	·0572	1·24	17·48
1·0	1·000	·0422	1·37	23·70
1·6	1·204	·0282	1·55	35·46

Several graphs were drawn:-

(i) Optical density versus concentration - This is not even approximately linear over any range. [Fig.18a]

(ii) Optical density versus $\log_{10}$(10× concentration) - This is nearer a straight line, but is rather inconvenient as it involves the logarithm of the concentration and does not pass through the origin. [Fig.18b]

(iii) Opacity (i.e.1/Transmittance) versus concentration - This is rather similar in shape, but involves the concentration instead of the logarithm of the concentration. It passes through the point (0,1). [Fig.18c]

Of these three graphs, method (iii) was chosen for subsequent work. An enlarged portion of this curve

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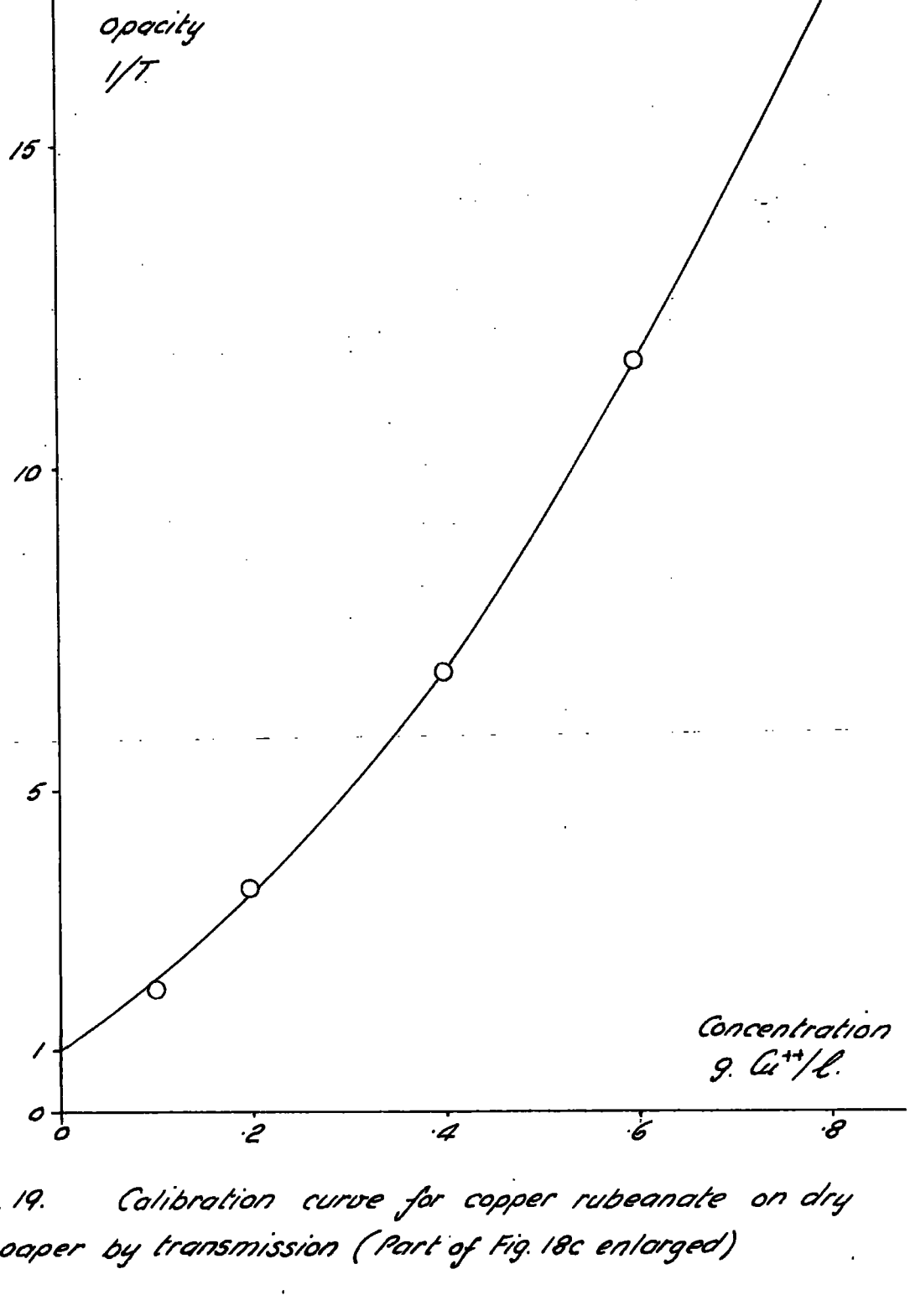


Fig. 19. Calibration curve for copper rubeanate on dry paper by transmission (Part of Fig. 18c enlarged)

involving the range of concentration from 0 to 0·8 g.Cu^{++}/ℓ is shown in Fig.19.

(c) Comparative suitability of dry and of transparent paper

The calibration curve obtained using transparent paper for transmission measurements has the advantage of being a straight line, which is not the case for any of the graphs drawn from measurements made on dry paper. However, measurements made on dry paper are at least as accurate as those made on transparent paper and can be made immediately the spots have been developed and dried.

All subsequent work involving transmission was therefore carried out on dry paper.

§ 13

COMPARISON OF REFLECTION AND TRANSMISSION

Before proceeding with the analysis of chromatographed spots, it seemed desirable to investigate the relative merits of transmission (dry) and reflection (dry). This was done by finding the standard deviations of the reflection and transmission readings at various concentrations. The change in measured concentration, corresponding to these deviations, was then found from the calibration curve.

For reflection measurements, the area measured was 2 x 2 mm. in the centre of each spot. For transmission the area chosen was 3 x 4 mm., since areas smaller than this would have been expected to show considerable variations on account of the non-uniformity of the paper. This area was also approximately the same as that used in the scanning of chromatograms (See § 18).

First, the standard deviation of the transmission and reflection readings was found as follows. Ten spots of concentration 1 g.Cu^{++}/ℓ were placed on a strip of paper. These were developed under identical conditions and each spot was measured both by reflection and by transmission. The standard deviations both for transmittance and for reflectance were then calculated. This was repeated at various concentrations, ten spots of each concentration being read both by reflection and by transmission. The results together with the standard deviations are shown in Tables 16 and 17. These tables also show the corresponding changes in measured concentration and the resulting probable relative error.

Tables overleaf.

Table 16

% Transmittance readings on non-chromatographed spots

No	Concentration g.Cu^{++}/l. 1	0·4	0·1	0·05
1	4·8	12·3	47·6	60·7
2	3·7	13·5	50·5	64·2
3	3·6	11·8	51·7	68·5
4	4·2	14·2	47·0	66·3
5	4·0	14·2	47·0	68·5
6	3·9	13·2	52·8	70·8
7	4·3	12·8	47·0	68·0
8	4·5	13·5	52·8	67·5
9	4·4	11·3	50·5	67·5
10	4·5	11·9	50·0	68·5
Mean % transmittance	4·2	12·9	49·7	67·0
Standard deviation	±0·36	± 0·97	±2·3	±2·6
Change in measured concentration g.Cu^{++}/l. From Figs.18c and 19	±0·059	±0·027	±0·0079	±0·0060
% Probable Relative error = $100 \times \frac{\text{Change in measured concentration}}{\text{concentration}}$	±5·9	±6·8	±7·9	±12

Table 17

% Reflectance readings on non-chromatographed spots

No	Concentration g.Cu^{++}/l.			
	1	0·4	0·1	0·05
1	18·3	36·8	57·6	66·1
2	18·4	37·4	57·9	67·5
3	18·7	37·2	57·6	70·0
4	18·7	37·5	58·3	66·8
5	18·7	37·3	58·4	69·5
6	18·3	36·8	59·0	68·5
7	18·8	38·0	59·1	69·0
8	19·7	37·5	58·9	69·1
9	19·2	38·1	58·5	68·7
10	19·2	37·2	59·1	69·1
Mean % reflectance	18·8	37·4	58·4	68·4
Standard deviation	±0·43	±0·41	±0·56	±1·2
Change in measured concentration g.Cu^{++}/l. From Fig.14	±0·043	±0·009	±0·0036	±0·005
% Probable Relative error = $100 \times \frac{\text{Change in measured concentration}}{\text{concentration}}$	±4·3	±2·2	±3·6	±10·0

Conclusion

Under the given experimental conditions, the reflection method is more precise than the transmittance method.

§ 14 - 16

CHROMATOGRAPHIC SEPARATION

§ 14

PRELIMINARY STUDY OF SOLVENTS

Copper and nickel are relatively easy to separate by chromatography, but considerable care is required to obtain reproducible results. A preliminary investigation was carried out to find solvents which would give spots of suitable R_f, shape, and size.

First, a number of solvent mixtures were tried, using the ascending technique described on page 55 . In each case 20 ml. of solvent were placed in a gas jar, and swilled round. The top was closed and the jar was placed on a bench away from draughts and from direct sunlight. The jar was allowed to stand for ½ to 1 hour, in order to saturate the air space. During this time, the solvent was swilled round the jar once or twice more to speed up the saturation of the air space. Meanwhile one inch wide paper was spotted with approximately 1 μl. of a solution containing 4 g. each of Cu^{++} and Ni^{++} per litre of 3 N HCl. This small volume was used for preliminary work as it reduced the time required for separation. The spot was then allowed to dry and the paper was placed in the gas jar. This was done by opening the cover slips slightly to leave a slit 1 mm. wide, sliding in the paper until it nearly touched the surface of the liquid in the gas jar, and then closing the cover slips to hold the paper in position. The paper was normally allowed to equilibrate in the solvent vapour for about half an hour, before lowering the paper into the solvent and thus starting the elution.

The following solvents were investigated*, as shown in Table 18.

Table 18

Preliminary investigation of solvents

No	Components	Proportions by volume (by wt. for solids)	Notes
1	Butanol, Conc.HCl, Water	Various	Good separations, uniform spots, with certain proportions
2	Butanol, Conc.HCl, Glycol	Various	Poorer separations than No.1
3	Butanol, Conc. HBr, Water	Various	No improvement on No.1
4	Butanol, Benzoylacetone 0·1 N HNO_3	50,50,0·5	-
5	Butanol, Acetic acid, Water	Various	Poor separations or badly shaped spots
6	Acetone, Chloroform	90, 10	
7	Dioxan, Acetic acid	90, 10	
8	Acetone, Conc.HCl, Water	90, 5, 5	Good separation. Fairly uniform spots
9	Ethyl methyl ketone, Conc.HCl, Water	Various	Good separations in many cases

*Most of these solvents are given in the Appendix to F. H. Pollard and J. F. W. McOmie "Chromatographic Methods of Inorganic Analysis" Butterworth, London 1953.

Of these solvents, the most promising appeared to be No.1 and Nos. 8 and 9. A detailed investigation was then carried out on the following solvents:-

Butanol, conc. HCl, water solvents, discussed in § 15

Ketone, conc. HCl, water solvents, discussed in § 16.

§ 15

DETAILED STUDY OF BUTANOL SOLVENTS

(a) Preliminary

The preliminary investigation suggested that butanol HCl mixtures produced well-shaped spots, but that the time required for separation was rather long. Accordingly, it seemed wiser to concentrate on descending chromatography with butanol HCl mixtures, in an attempt to produce good separations and really well-shaped circular spots, suitable for a single reading method of measurement.

(b) Solvents investigated

Numerous butanol, conc. HCl, water mixtures of differing compositions were investigated. The most convenient way of preparing these was to shake butanol with dilute HCl and to separate off the organic layer for use. Stronger HCl mixtures produced greater separation, but tended to weaken and discolour the paper. The organic layer of butanol 3 N HCl was chosen as the most suitable solvent. This produced a complete separation by descending chromatography in 24 hours, and did not weaken or discolour the paper.

(c) Modification in procedure

The procedure was modified in various ways:-

(i) Grade of paper. [Whatman papers were used throughout.] Papers Nos. 3, 7, 32, 42, 44 were observed to have a slightly reticulated surface and were not investigated. The following papers were compared as shown in Table 19.

Table 19 overleaf.

Table 19

Comparison of Whatman filter papers with butanol 3 N HCl (Spots of Cu^{++})

No. of paper	Speed	Type of spot
4	Fast	Diffuse
1	Medium	Less diffuse
2	Medium/slow	Fairly compact, and of good shape
20	Slow	Compact

Paper No.2 was used for all work. It seemed doubtful whether the slight improvement in compactness of No.20 would be advantageous.

(ii) Time and method of equilibration - Paper, wet with the solvent, was hung round the inside of the tank, in order to speed up the saturation of the air space. 24 hours was long enough to ensure saturation. Fanning of the atmosphere by a small fan did not improve the separation in any way. Very long periods of saturation (e.g. a week) were found to alter slightly the R_f values, and to produce some discolouration.

(iii) Temperature - The effect of varying the temperature was not investigated, but all operations were carried out at about 22°C. in the constant temperature cabinet. [See § 2]

(iv) Time of drying of spot - The initial spots were dried for varying times from one minute to one hour. No difference in the R_f or shape of the spots was observed as a result of different drying times.

(v) Humidification - The spots were dried, and the papers were then humidified by hanging in a tank containing a saturated solution of a particular salt for a week. No differences were noted as a result of different degrees of humidification of the paper.

(vi) Volume of spot - Spots of the same metal content but of volumes varying from 1µl. to 10µl. were applied to a sheet of paper which was eluted for 24 hours. Neither very small nor very large initial spots appeared to be particularly

conducive to the formation of chromatographed spots of good shape.

(d) Summary of method

Spots of Cu^{++} and Ni^{++} in 3 N HCl (of volume 2·64 μl.) were placed on the paper 6·5 cm. from the shorter edge of the paper. After drying, the sheets were placed in an empty tank lined with paper, with the paper dipping into a trough. The organic layer of butanol 3 N HCl was used both for saturation and for elution. 250 ml. of the solvent were placed in the tank, in such a way as to wet the paper lining the tank. The lid was then closed and equilibration allowed to take place for 24 hours. 40 ml. of the solvent were then placed in the trough through a hole in the lid and elution was allowed to take place for 24 hours, after which time the solvent had run off the end of the paper. The papers were then removed and allowed to dry in the cabinet for 2 hours before development. The resulting spots were of good 'circular' shape. Their dimensions are given in Table 20.

Table 20

Dimensions of chromatographed spots using the solvent butanol 3 N HCl [Time of elution 24 hours.]

Concentration g.Cu^{++}/l. [Volume 2·64 μl.]	Distance run by copper spot cm.	Concentration g.Ni^{++}/l. [Volume 2·64 μl.]	Distance run by nickel spot cm.
1·0	7·2 - 9·1	1·0	4·4 - 6·0
0·5	7·4 - 9·2	0·5	4·5 - 6·1
0·25	7·5 - 9·0	0·25	4·7 6·1

§ 16

DETAILED STUDY OF KETONE SOLVENTS

(a) Preliminary

Ketone solvents ran rapidly compared with butanol solvents, but the resulting chromatograms appeared to be more affected by slight variations in procedure. The object of the following work was to find a method of producing good 'rectangular' spots on strips of paper 2 cm. wide, suitable for scanning.

(b) Solvents investigated

(i) Methyl ethyl ketone, conc. HCl, water solvents were investigated, and the solvent methyl ethyl ketone 90, conc. HCl 10* appeared, at first, to be very promising. A complete separation was produced in less than half an hour. The spot of copper, however, underwant a change which will be termed 'straight line formation'. This is a tendency for the initial spot to change into a line-shaped spot at right angles to the direction of flow, during the first few minutes of elution. This line-shaped spot gradually changed back into a round spot, but the time taken for this change varied greatly as a result of small differences in procedure which were difficult to control. It seemed best to avoid this straight line formation altogether, and it was found that this could be done by omitting the usual period of equilibration of the paper in the solvent vapour and replacing it by equilibrating the paper in HCl vapour for half an hour. This procedure produced a good separation and well shaped spots.

(ii) Methyl propyl ketone, conc. HCl solvents were investigated but were found to be very prone to straight line formation. Acetone, conc. HCl solvents appeared less prone to straight line formation, but ran a little too rapidly for closely reproducible chromatography.

* all solvent compositions are given by volume.

(iii) In an attempt to modify the acetone conc.HCl solvents so as to slow down the rate of flow, butanol was added. The solvent system acetone, butanol, conc. HCl, water was investigated. The proportion of each component in the mixture was varied in turn. The most suitable proportions were found to be acetone 50, butanol 46, conc. HCl 10, water 7·5. This produced a good separation and almost perfect rectangular spots, which did not suffer from straight line formation. This solvent was used in preference to methyl ethyl ketone 90, conc. HCl 10 as it was found to be easier to obtain reproducible results. No equilibration of the paper in the solvent vapour or in HCl vapour was necessary.

(c) Modification in procedure

(i) Shape of initial spot - The best results were obtained by placing three spots each of volume 2·64µl. side by side on the starting line. This produced an almost rectangular initial spot.

(ii) Starting point position - This was varied from 1 to 8 cm. 3 cm. was chosen as the most suitable distance.

(iii) Time of elution - 30 minutes at 23°C. or 45 minutes at 20°C. produced complete separation. Longer times resulted in longer spots which could not be scanned in the transmission adaptor.

(d) Summary of method

Three 2·64µl. spots were placed on the starting line, 3 cm. from the end of a 2 cm. wide strip of paper. After drying for 10 minutes, the papers were lowered into a gas jar the air space of which had been allowed to saturate with 20 ml. of the solvent mixture acetone 50, butanol 46, conc. HCl 10, water 7·5 for at least an hour. Elution was started at once and allowed to proceed for 30 minutes at 23°C., after which the papers were dried for one hour before development. The dimensions and the R_f values of the chromatographed spots are shown in Tables 21 and 22 respectively.

Table 21

Dimensions of chromatographed spots of copper [Time of elution 30 minutes, using the solvent acetone 50, butanol 46, conc. HCl 10, water 7·5]

Concentration g.Cu^{++}/l. [Volume 3 x 2·64µl.]	Distance run by copper spot cm.
0·1	1·6 - 3·2
0·25	1·6 - 3·3
0·40	1·6 - 3·4

The front of the nickel spot moved only 0·8 cm. from the starting line. The R_f values are shown below in Table 22.

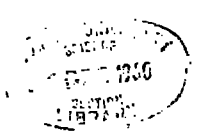
Table 22

Approximate R_f values of copper and nickel using the solvent acetone 50, butanol 46, conc. HCl 10, water 7·5

Metal	R_f based on centre of spot
Nickel	0·02
Copper	0·41

§ 17 - 19

PHOTOMETRY OF CHROMATOGRAPHED SPOTS



§ 17

PRELIMINARY

The study of photometric estimation of non-chromatographed spots (§ 3 - 13) indicated two methods of estimation which might well be applied to chromatographed spots. These were:-

(i) A single reading reflection method

(ii) A transmission method suitable for scanning

The work done on solvents (§ 14 - 16) showed that the following produced good separations.

(i) Butanol 3 N HCl giving round spots on broad sheets of paper by descending chromatography.

(ii) Acetone 50, butanol 46, conc. HCl 10, water 7·5 giving rectangular spots on thin strips of paper by ascending chromatography.

It was therefore decided to make a comparison between:-

(i) The single reading reflection method applied to a series of round spots of varying concentrations which had been eluted on the same sheet of paper by butanol 3 N HCl.

(ii) The scanning transmission method applied to rectangular spots which had been eluted on a series of thin strips of paper by the solvent acetone 50, butanol 46, conc. HCl 10, water 7·5.

The estimation of chromatographed spots was limited to that of copper alone.

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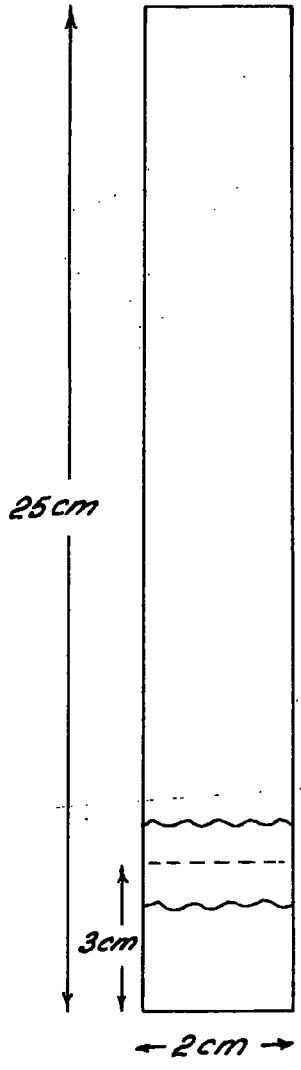

Fig. 20. Preparation of chromatogram on narrow strip

§ 18

SCANNING TRANSMISSION METHOD

(a) Description of method

(i) Application of spots to paper - Three spots each of volume 2·64 µl. were applied to the starting line of a strip of paper 2 cm. wide to produce a rectangular spot, as shown in Fig.20. Three standards, one blank, and one unknown were prepared on five strips of paper. The spots were dried for at least 10 minutes.

(ii) Elution - 20 ml. portions of the solvent acetone 50, butanol 46, conc. HCl 10, water 7·5 were placed in each of five gas jars. The gas jars were closed with cover slips (§ 2) and allowed to saturate for an hour. The papers were then added, one to each gas jar, by opening the cover slips 1 mm., sliding in the papers until they just touched the solvent, and closing the slips so as to grip the paper and seal the gas jar. The papers were not allowed to equilibrate in the solvent vapour, as elution was started at once. The papers were eluted for 30 minutes at 23°C., removed, and dried for one hour.

(iii) Development - The papers were developed by hanging in ammonia vapour for half a minute, immersing in 0·1% alcoholic rubeanic acid for one minute, hanging again in ammonia vapour for half a minute, and finally washing in alcohol for half a minute. The papers were then dried for at least five minutes before measuring.

(iv) Colour measurement - The copper spots were cut out and each was placed, in turn, in the measure compartment of the scanning transmission adaptor described in § 2.

The window measured 1·9 cm. x 3 cm. The indirect standard method described in § 7 was used. Each of the spots was scanned in turn (Tables 25 - 28), using a grey filter as a standard in each case. A scanning slit 2 mm. wide was used. The mean transmittance of the blank spot relative to the filter was also found by scanning as shown in Table 24 and Fig.21. This enabled each of the transmittance readings on the spots to be expressed relative to the mean transmittance of the blank spot. All readings were taken at 660 mμ with a spectrophotometer slit width of 0·3 mm.

(v) Calculation - The readings of transmittance relative to the mean transmittance of the blank spot are referred to as 'absolute transmittance' in the following tables. A table of conversion of transmittance to concentration (Table 23) was prepared from Figs.18c and 19, and this enabled the Cu^{++} concentration in each element to be obtained as shown in Tables 25 - 28. Graphs showing the distribution of material of each spot were drawn as shown in Figs.22 - 25; the area under the curve being a measure of the total concentration of copper in the initial solution.

A calibration graph of the area under the scanning curves (shown in Figs. 22 - 25) versus the initial spot concentration was drawn, as shown in Fig.26, from which the concentration of the unknown was interpolated as shown in Table 29.

A calibration curve of the maximum concentration of Cu^{++} in any element versus the initial spot concentration, was also drawn, as shown in Fig.27 from which the concentration of the unknown was found by interpolation, as shown in Table 30.

(b) Tables of results

Three complete sets of chromatograms were run and these are referred to as Series 1, 2 and 3 respectively. The complete working is shown for Series 1 in Tables 24 - 30 together with results of Series 2 and 3 summarised in Tables 31 and 32.

Table 23

Conversion of % transmittance (100T) to concentration of Cu^{++} (c) in g.Cu^{++}/l. From Figs.18c and 19

100T	c	100T	c	100T	c	100T	c
7·0	·68	9·4	·56	13·6	·43	28	·21
·1	·68	·5	·56	·8	·42	29	·20
·2	·67	·6	·55	14·0	·41	30	·19
·3	·66	·7	·55	·5	·40	32	·18
·4	·66	·8	·54	15·0	·39	34	·17
·5	·65	·9	·54	·5	·38	36	·16
·6	·64	10·0	·53	16·0	·37	38	·14
·7	·64	·2	·53	·5	·36	40	·13
·8	·63	·4	·52	17·0	·35	42	·12
·9	·63	·6	·51	·5	·34	44	·11
8·0	·62	·8	·51	18·0	·33	46	·11
·1	·62	11·0	·50	·5	·32	48	·10
·2	·61	·2	·49	19·0	·32	50	·09
·3	·61	·4	·49	·5	·31	55	·07
·4	·61	·6	·48	20·0	·30	60	·06
·5	·60	·8	·47	·5	·29	65	·05
·6	·60	12·0	·47	21·0	·29	70	·04
·7	·59	·2	·46	·5	·28	75	·03
·8	·59	·4	·46	22·0	·27	80	·02
·9	·58	·6	·45	23	·26	85	·02
9·0	·58	·8	·45	24	·25	90	·01
·1	·57	13·0	·44	25	·24	95	·01
·2	·57	·2	·44	26	·23	100	0
·3	·57	·4	·43	27	·22		

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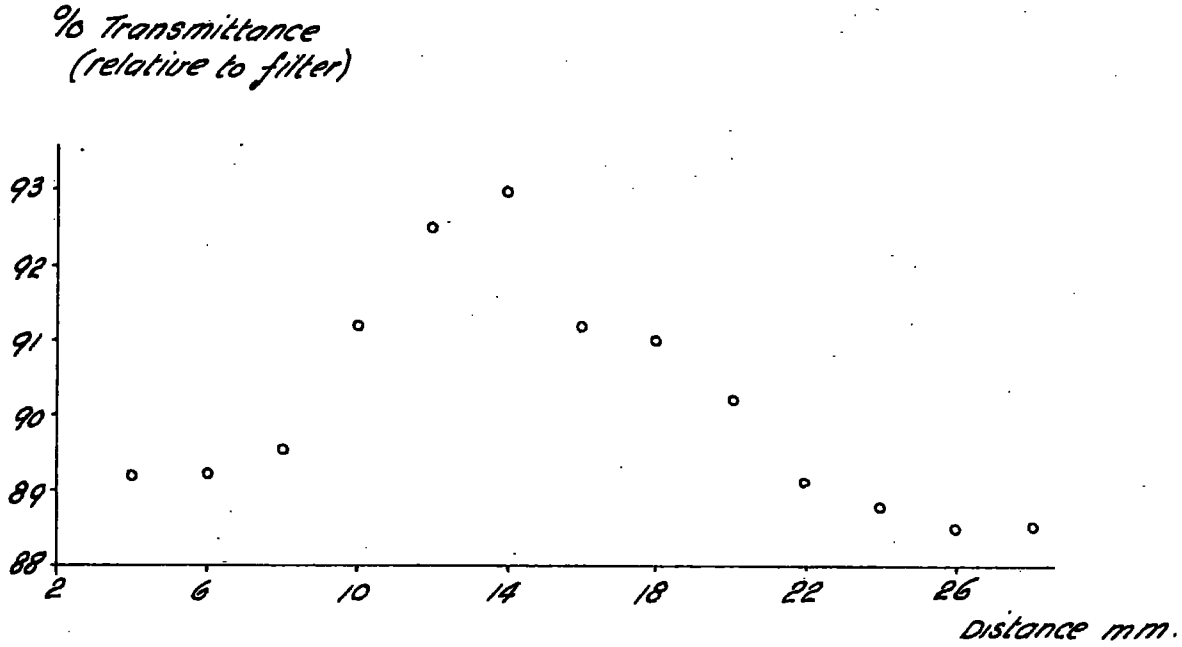

Fig. 21. Determination of mean transmittance of blank spot — Series I

Table 24

Determination of mean transmittance of the blank spot - Series 1

Distance mm.	% transmittance relative to filter
4	89·2
6	89·2
8	89·5
10	91·2
12	92·5
14	93·0
16	91·2
18	91·0
20	90·2
22	89·1
24	88·8
26	88·5
28	88·5

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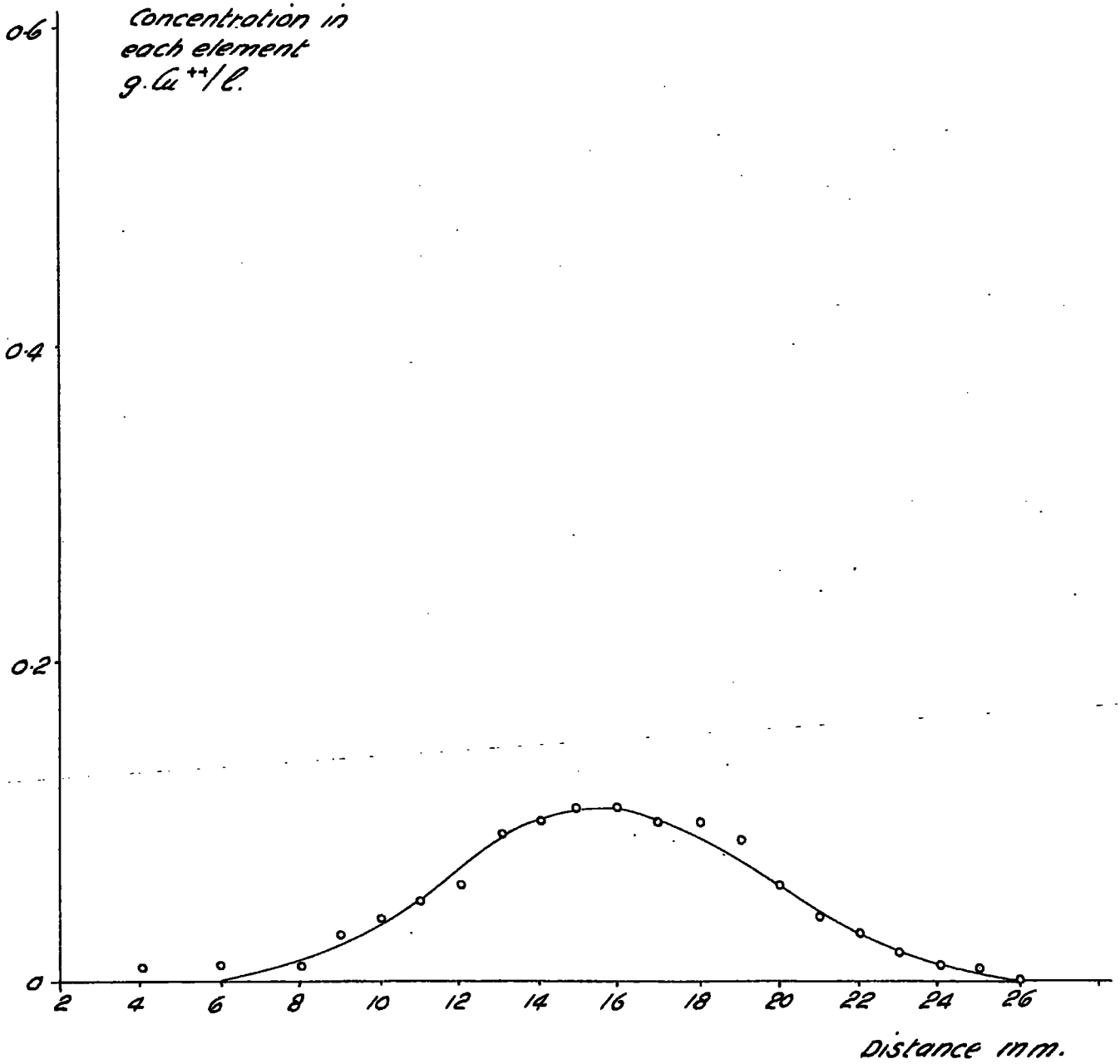

Fig. 22 Scanning curve of standard spot A – Series I

Table 25

Scanning of spot A - Series 1 See page 112

Distance mm.	% Transmittance reading	Absolute % transmittance	Concentration in each element g.Cu^{++}/l.
2	87·0	97	0
3	-	-	-
4	86·0	96	·01
5	-	-	-
6	81·4	90	·01
7	-	-	-
8	79·5	88	·01
9	69·0	77	·03
10	63·7	71	·04
11	59·7	66	·05
12	53·9	60	·06
13	46·8	52	·09
14	43·7	49	·10
15	42·3	47	·11
16	41·8	47	·11
17	42·7	48	·10
18	43·8	49	·10
19	45·9	51	·09
20	52·4	58	·06
21	60·8	68	·04
22	69·2	77	·03
23	76·3	85	·02
24	81·6	91	·01
25	85·6	95	·01
26	88·2	98	0
27	90·2	-	-
28	92·0	-	-

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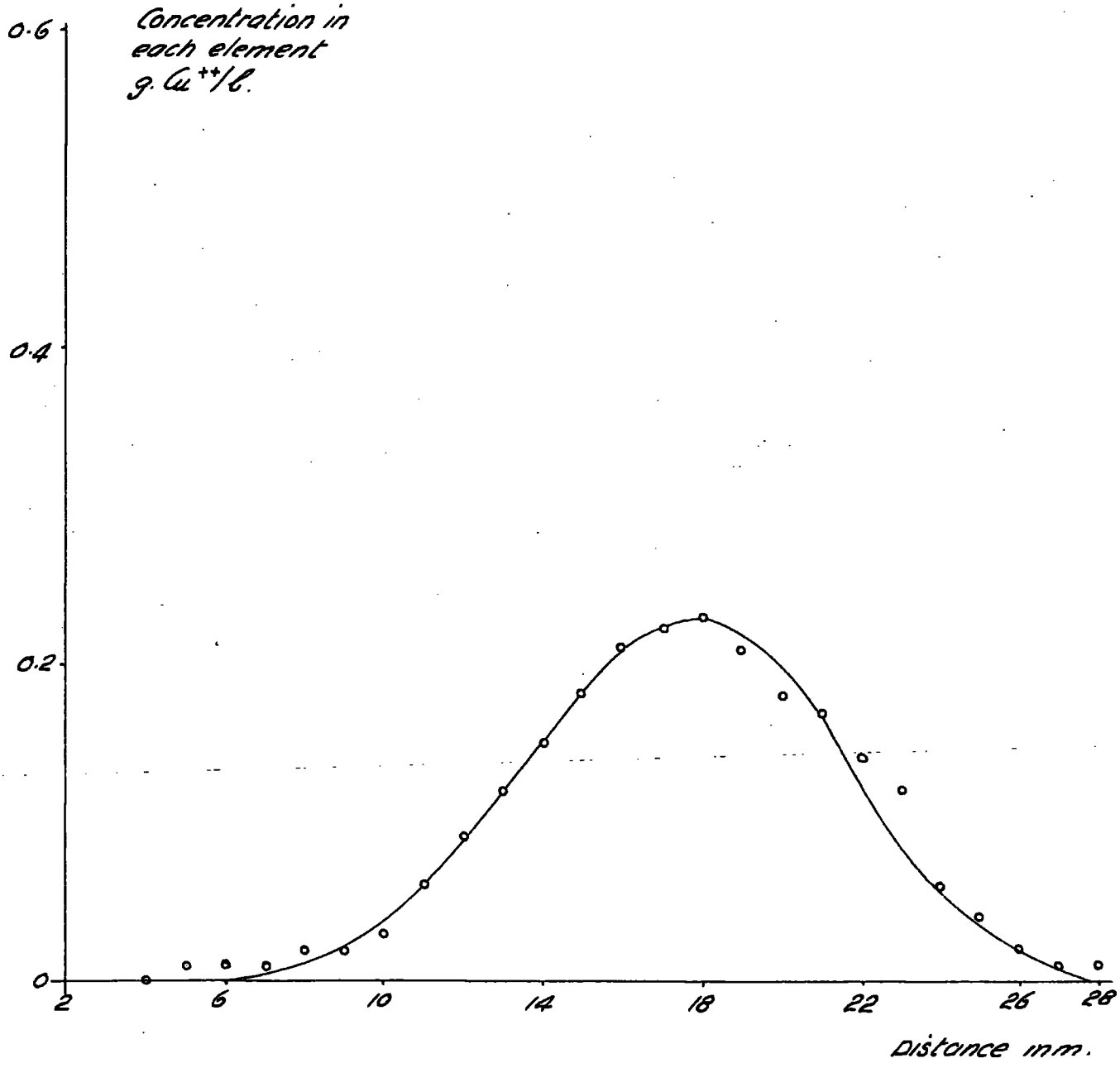

Fig. 23 Scanning curve of standard spot B – Series I

Table 26

Scanning of spot B - Series 1 See page 112

Distance mm.	% Transmittance reading	Absolute % transmittance	Concentration in each element g.Cu^{++}/l.
2	89·0	99	0
3	88·8	99	0
4	88·2	98	0
5	86·8	97	·01
6	83·8	93	·01
7	80·6	90	·01
8	76·7	85	·02
9	75·0	83	·02
10	68·1	76	·03
11	55·3	62	·06
12	45·8	51	·09
13	38·4	43	·12
14	33·2	37	·15
15	28·4	32	·18
16	25·3	28	·21
17	24·0	27	·22
18	23·6	26	·23
19	25·3	28	·21
20	28·2	31	·18
21	31·8	35	·17
22	35·1	39	·14
23	38·1	42	·12
24	54·8	61	·06
25	64·1	71	·04
26	72·2	80	·02
27	79·1	88	·01
28	83·8	93	·01

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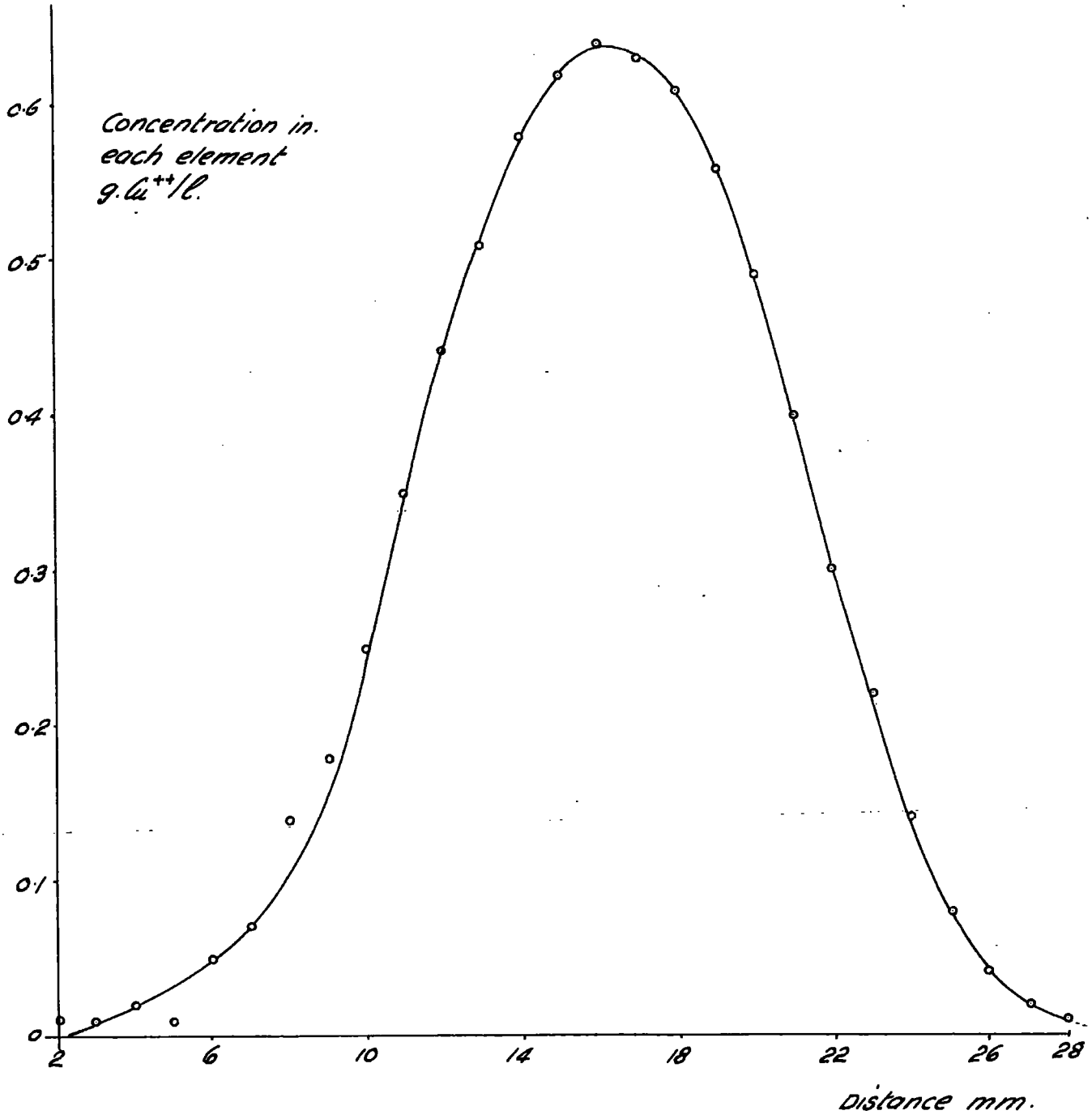

Fig. 24. Scanning curve of standard spot C – Series I

Table 27

Scanning of spot C - Series 1 See page 112

Distance mm.	% Transmittance reading	Absolute % transmittance	Concentration in each element g.Cu^{++}/ℓ.
2	83·8	93	·01
3	82·4	92	·01
4	77·0	86	·02
5	79·6	89	·01
6	58·0	65	·05
7	48·9	54	·07
8	34·8	39	·14
9	28·6	32	·18
10	21·2	24	·25
11	15·2	16·9	·35
12	11·8	13·1	·44
13	9·5	10·5	·51
14	8·1	9·00	·58
15	7·2	8·00	·62
16	6·9	7·66	·64
17	7·1	7·89	·63
18	7·5	8·34	·61
19	8·5	9·43	·56
20	10·2	11·3	·49
21	13·2	14·6	·40
22	17·8	19·8	·30
23	24·4	27	·22
24	34·3	38	·14
25	47·1	52	·08
26	60·6	68	·04
27	80·3	78	·02
28	81·9	90	·01

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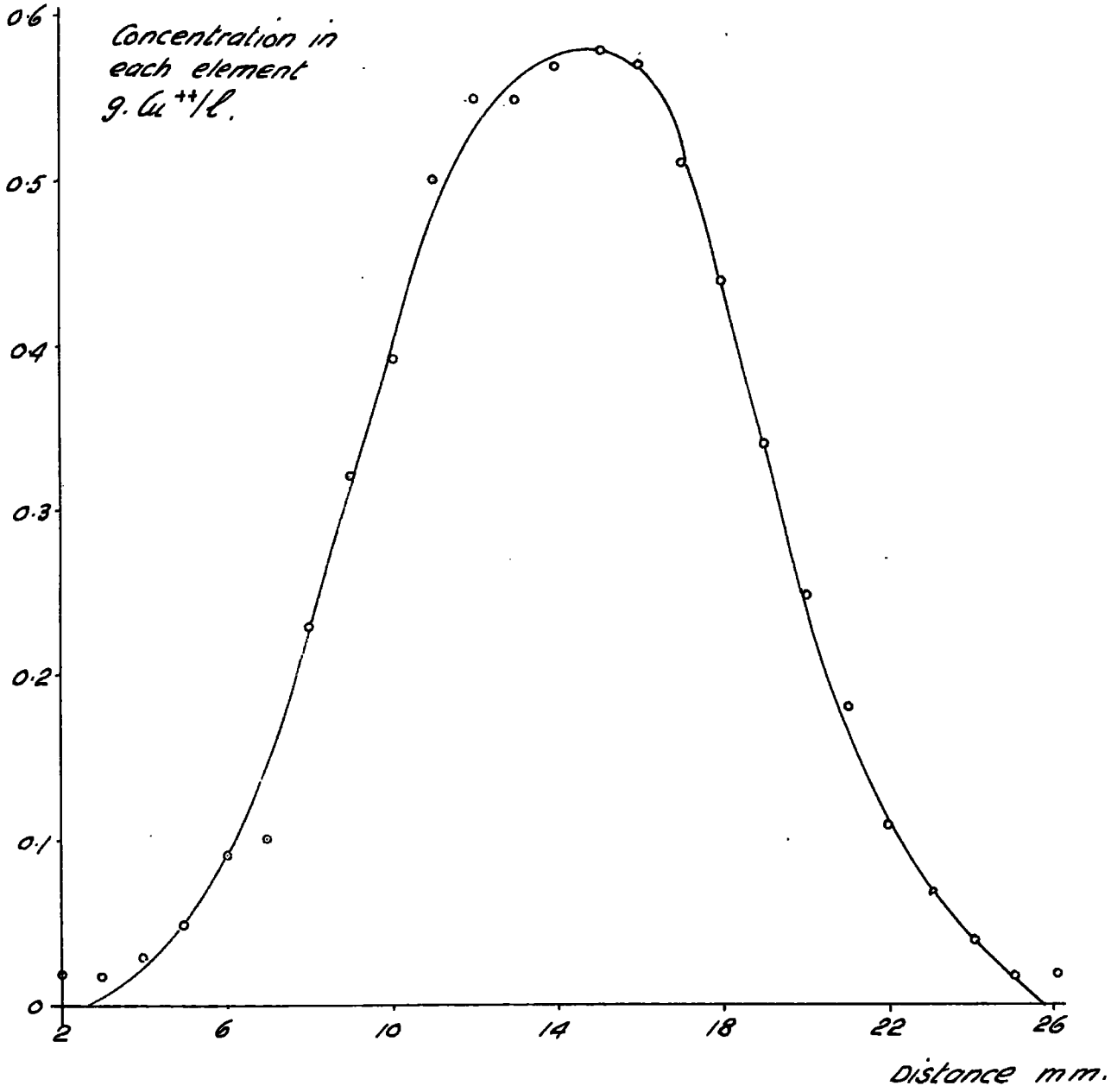

Fig. 25 Scanning curve of 'unknown' spot D - Series I

Table 28

Scanning of spot D - Series 1 See page 112

Distance mm.	% Transmittance reading	Absolute % transmittance	Concentration of each element g.Cu^{++}/ℓ.
2	74·3	82	·02
3	73·4	81	·02
4	66·7	74	·03
5	57·1	63	·05
6	45·8	51	·09
7	43·2	48	·10
8	23·8	26	·23
9	16·8	18·7	·32
10	13·5	15.0	·39
11	10·0	11·1	·50
12	8·7	9·7	·55
13	8·6	9.6	·55
14	8·4	9·3	·57
15	8·1	9·0	·58
16	8·4	9·3	·57
17	9·7	10·8	·51
18	12·0	13·3	·44
19	15·8	17·5	·34
20	21·8	24	·25
21	29·6	33	·18
22	39·0	43	·11
23	50·0	55	·07
24	63·0	70	·04
25	72·0	80	·02
26	78·7	87	·02
27	83·1	92	-
28	87·0	96	-

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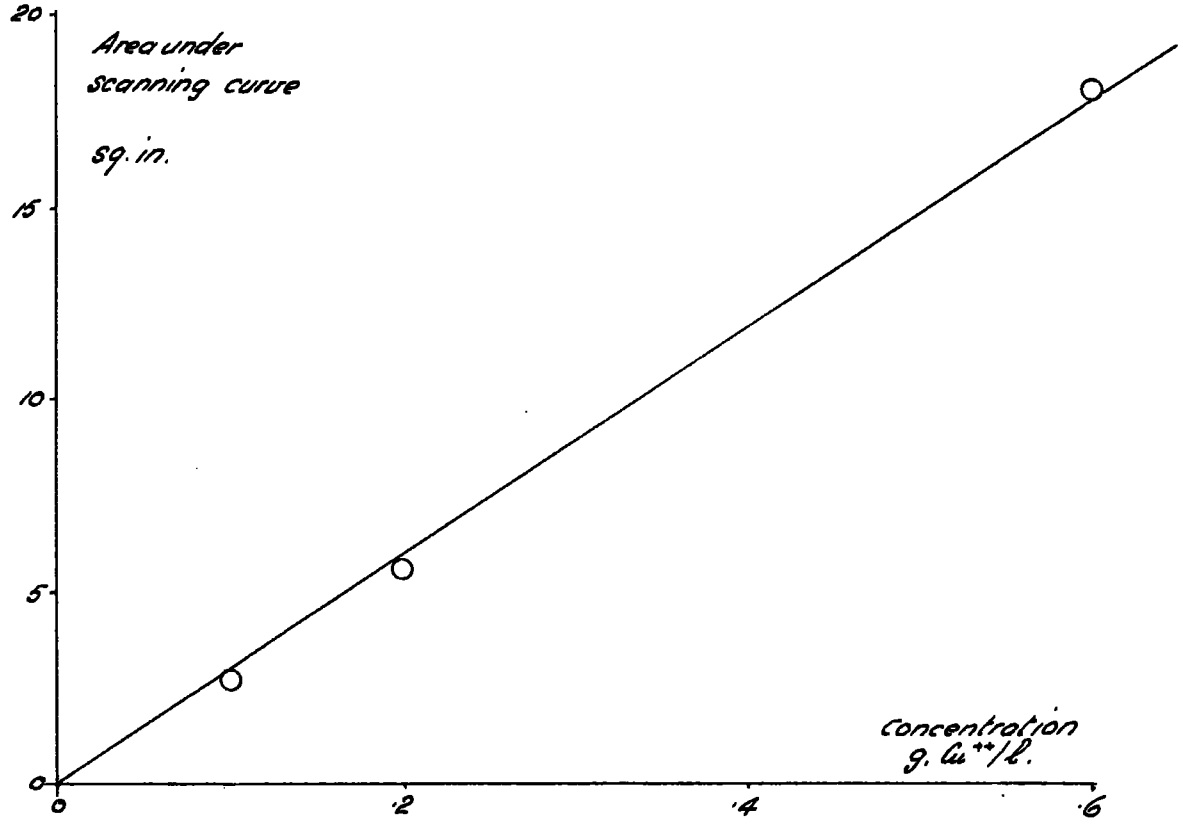

Fig. 26 Calibration curve, scanning transmittance method - Series I

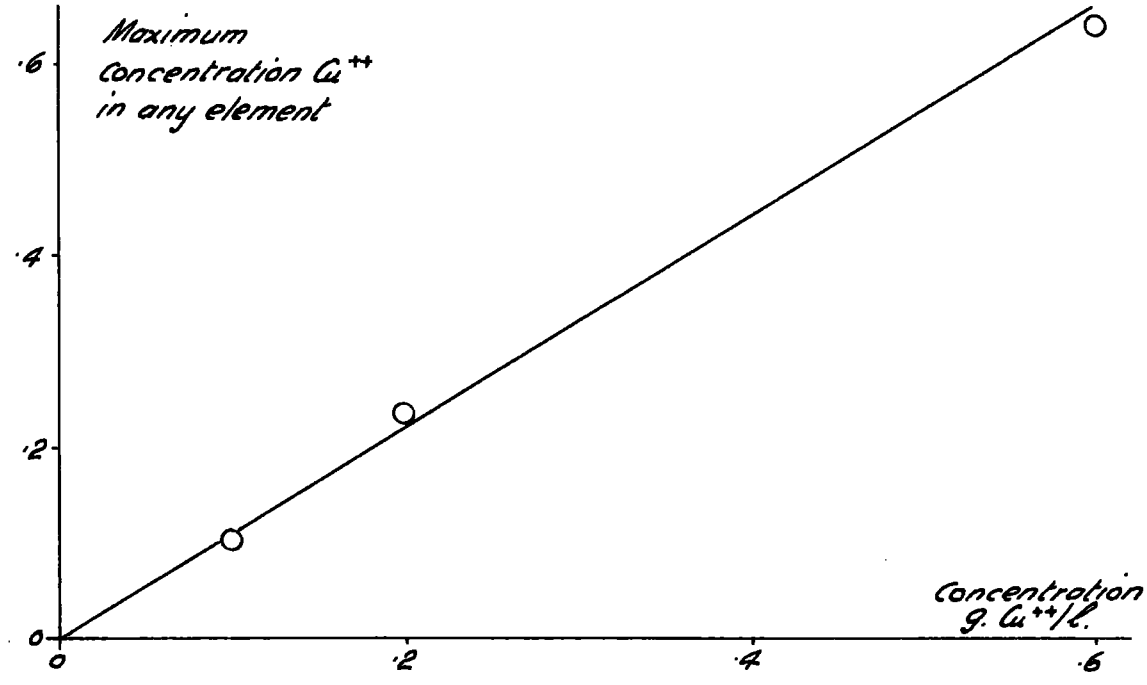

Fig. 27 Calibration curve, minimum transmittance method - Series I

Table 29

Summary of estimation of spot from area under scanning curve - Series 1

No.of spot	Area under curve sq.in.	Concentration initial spot solution g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l.
A	2·61	0·1	-
B standards	5·79	0·2	-
C	18·20	0·6	-
D 'unknown'	16·20	0·5	0·54

Table 30

Summary of estimation of spot from minimum transmittance - Series 1

No. of spot	Minimum % transmittance	Maximum concentration in any element g.Cu^{++}/l.	Concentration initial spot solution g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l.
A standards	46·4	0·105	0·1	-
B standards	26·2	0·235	0·2	-
C standards	7.66	0·645	0·6	-
D 'unknown'	9·0	0·575	0·5	0·52

Table 31

Summary of estimation of spot from area under scanning curve - Series 2 and 3

No.of spot	Area under curve sq.in.	Concentration initial spot solution g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l.
Series 2			
1 } standards	2·76	0·1	-
2 } standards	14·14	0·4	-
3 unknown	7·87	0·25	0.235
Series 3			
1 } standards	3·68	0·1	-
2 } standards	11·70	0·4	-
3 unknown	8·04	0·25	0·26

Table 32

Summary of estimation of spot from minimum transmittance - Series 2 and 3

No. of spot	Minimum % transmittance	Maximum concentration in any element g.Cu^{++}/l.	Concentration initial spot solution g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l.
Series 2				
1 } standards	45.0	0.11	0.1	-
2 } standards	10.4	0.52	0.4	-
3 'unknown'	18.7	0.32	0.25	0.26
Series 3				
1 } standards	44.0	0.11	0.1	-
2 } standards	11.4	0.49	0.4	-
3 'unknown'	18.5	0.32	0.25	0.265

(c) Discussion of results of transmission method

The results of the above work are summarised below in Table 33.

Table 33

Summary of results of transmission work

Series No.	Concentration of 'unknown' g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l. By area under scanning curve method	By minimum transmittance method
1	0·5	0·54	0·52
2	0·25	0·23(5)	0·26
3	0·25	0·26	0·26(5)

The mean relative error of 3 readings is about $\pm$ 5% both by the area and by the minimum transmittance methods of estimation. There would not appear to be any justification, in this particular case, for the additional labour involved in scanning.

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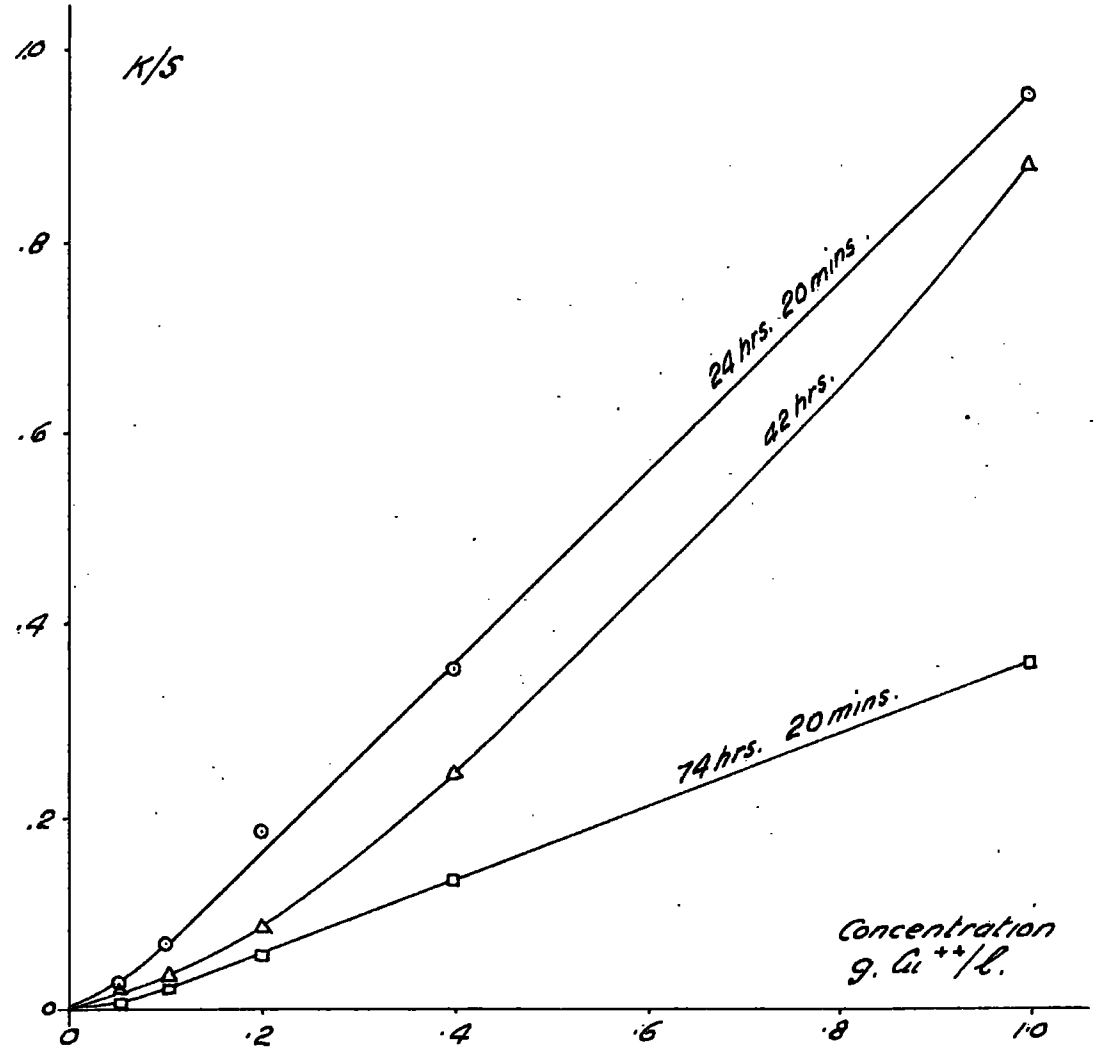

Fig. 28. Effect of time of elution on shape of calibration curve

§ 19

SINGLE READING REFLECTION METHOD

(a) Choice of the most suitable time of elution

Spots of various concentrations were placed on the starting line of a large rectangular sheet of paper and eluted for 24 hours 20 minutes by descending chromatography, using butanol 3 N HCl as a solvent. This was repeated using different times of elution. The results are shown in Table 34 and in Fig.28.

Table 34

% Reflectance (100R) and K/S values for differing concentrations and times of elution

Concentration of initial spot g.Cu^{++}/ℓ	Time of elution 24hrs.20min. 100R	K/S	42hrs. 100R	K/S	74hrs.20min. 100R	K/S
1.0	27.5	0·956	28·8	0·880	43·8	0·360
0·4	44.1	0·354	50·3	0.246	59·8	0·135
0·2	55·2	0·182	66·3	0·086	71·8	0·055
0·1	69·2	0·069	76·8	0·035	82·5	0·019
0.05	78·3	0·030	81·8	0·020	90·0	0·006

From these results it is clear that long elution times which produce shallow K/S versus concentration curves are to be avoided. The K/S versus concentration curve is almost a straight line from 0·1 to 1·0 g.Cu^{++}/ℓ, when the time of elution is 24 hours 20 minutes. Since this time was sufficient to produce complete separation of nickel and copper it was chosen for subsequent work.

(b) Description of method

(i) Application of spots to paper - 2·64 µl. spots were applied to the starting line of a sheet of paper 27 cm. wide and 33 cm. long. The starting line was 7·5 cm. from the shorter edge of the paper. Two types of sheet were prepared: 'A' sheets in which the copper concentration was varied (which contained three unknowns, four standards, and one blank) and 'B' sheets containing spots of the same copper content, but of varying concentration of nickel and other metals. The object of the 'B' sheets was to investigate the change in measured concentration of copper brought about by differing quantities of other metals. Table 35 gives details of the spots placed on the sheets of paper.

Table 35 overleaf

Table 35

Details of spots placed on sheets of paper (Volume of spot 2·64 µl.; concentration in g./l.)

Sheets 'A'								
No.	A1	A2	A3	A4	A5	A6	A7	Blank
Cu^{++} concentration	1·0	0·8	0·1	0·5	0·6	0·25	0·2	-
Ni^{++} concentration	-	0·5	-	0·5	-	0·5	-	-

A1, A3, A5 and A7 are standards

Sheets 'B'								
No.	B1	B2	B3	B4	B5	B6	B7	Blank
Cu^{++} concentration	0·5	0·5	0·5	0·5	0·5	0·5	0·5	-
Ni^{++} concentration	4	-	2	.25	1	·5	-	-
Other metals	-	-	-	-	-	-	*	-

* No Ni^{++} but mercuric Hg 2 g./l.
+Cd 1 g./l.
+Bi 1 g./l.

(ii) Elution - The sheets were eluted for 24 hours by descending chromatography, after being allowed to equilibrate in the solvent vapour for 24 hours. The papers were then dried and the regions containing the copper spots were cut out and developed.

(iii) Development - The papers were developed by hanging in ammonia vapour for half a minute, immersing in 0·1% alcoholic rubeanic acid for one minute, hanging again in ammonia vapour for half a minute, and finally washing in alcohol for half a minute. The papers were then dried for

at least 5 minutes before measuring.

(iv) Colour measurement - A circle of paper containing the spot to be measured was cut out and placed in the measure holder of the reflection attachment. The blank spot was placed in the standard holder. Six sheets of white paper were used as a background in both cases. The reflectance of each spot was measured at 660 mμ using a spectrophotometer slit width of 0·1 mm.

(c) Tables of results

Four 'A' sheets and two 'B' sheets were run. The results are shown in Tables 36 - 38 and in Figs. 29 - 31.

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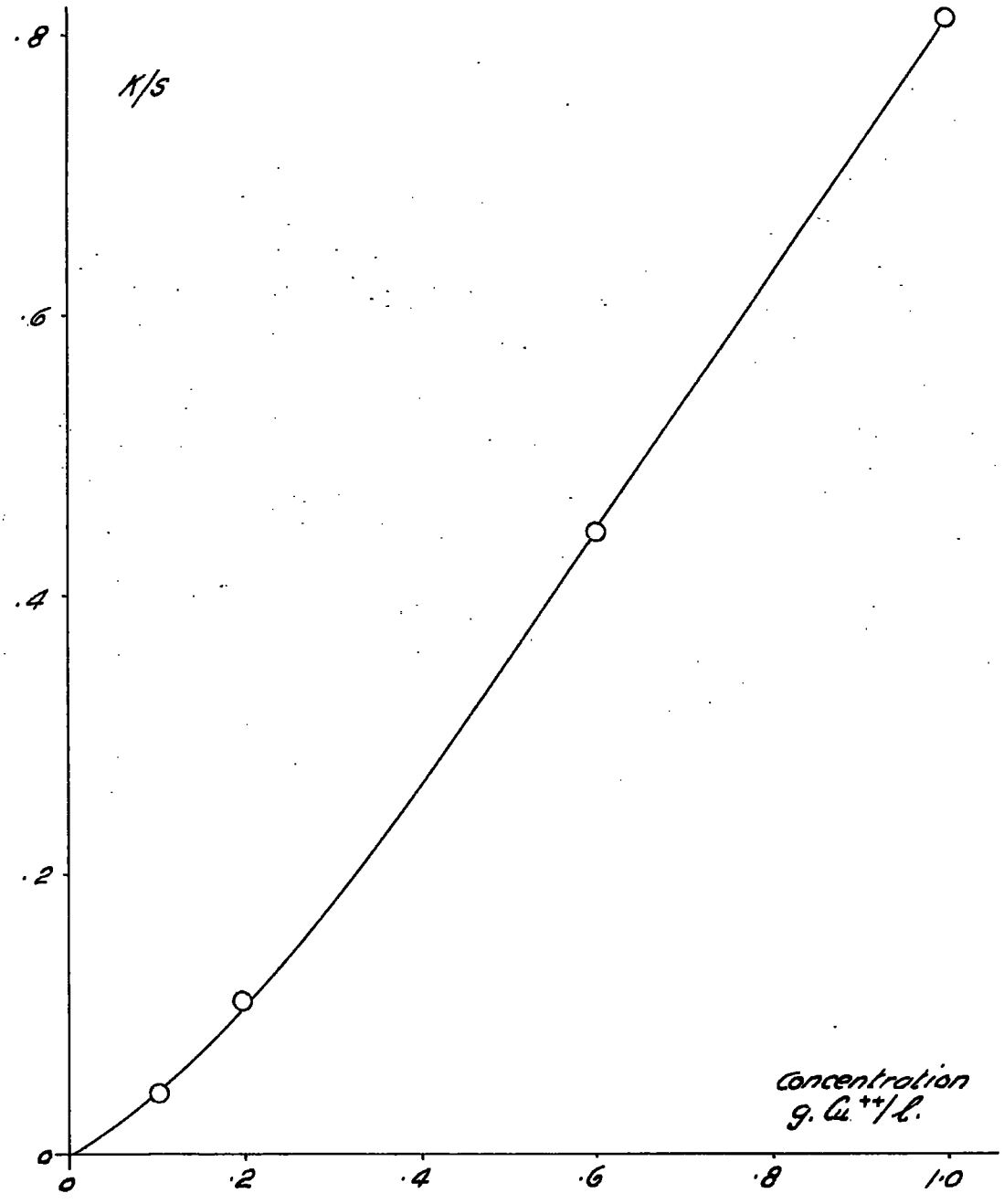

Fig. 29. Calibration curve, reflection method — 1st. set.

Table 36

1st Set

One 'A' sheet and one 'B' sheet run simultaneously [See Table 35]

Temperature of elution 23°C. Time of elution 24 hours.
Volume of spot 2·64µl.

No.of spot	% Reflectance at centre of spot	K/S	Concentration g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l.
A1	30·1	0·812	1·0	
A2	35·7	0·579	0·8	0·75
A3	74·5	0.044	0·1	
A4	45·1	0·334	0·5	0·48
A5	40·2	0·445	0·6	
A6	56·7	0·165	0·25	0·27
A7	62·8	0·110	0·2	
B1	47·3	0·294	0·5	0·43
B2	45·0	0·336	0·5	0·48
B3	45·6	0·324	0·5	0·47
B4	45·0	0·336	0·5	0·48
B5	41·3	0·417	0·5	0·57
B6	42·6	0·387	0·5	0·54
B7	43·1	0·376	0·5	0·52

* Read from curve of 'A' sheet

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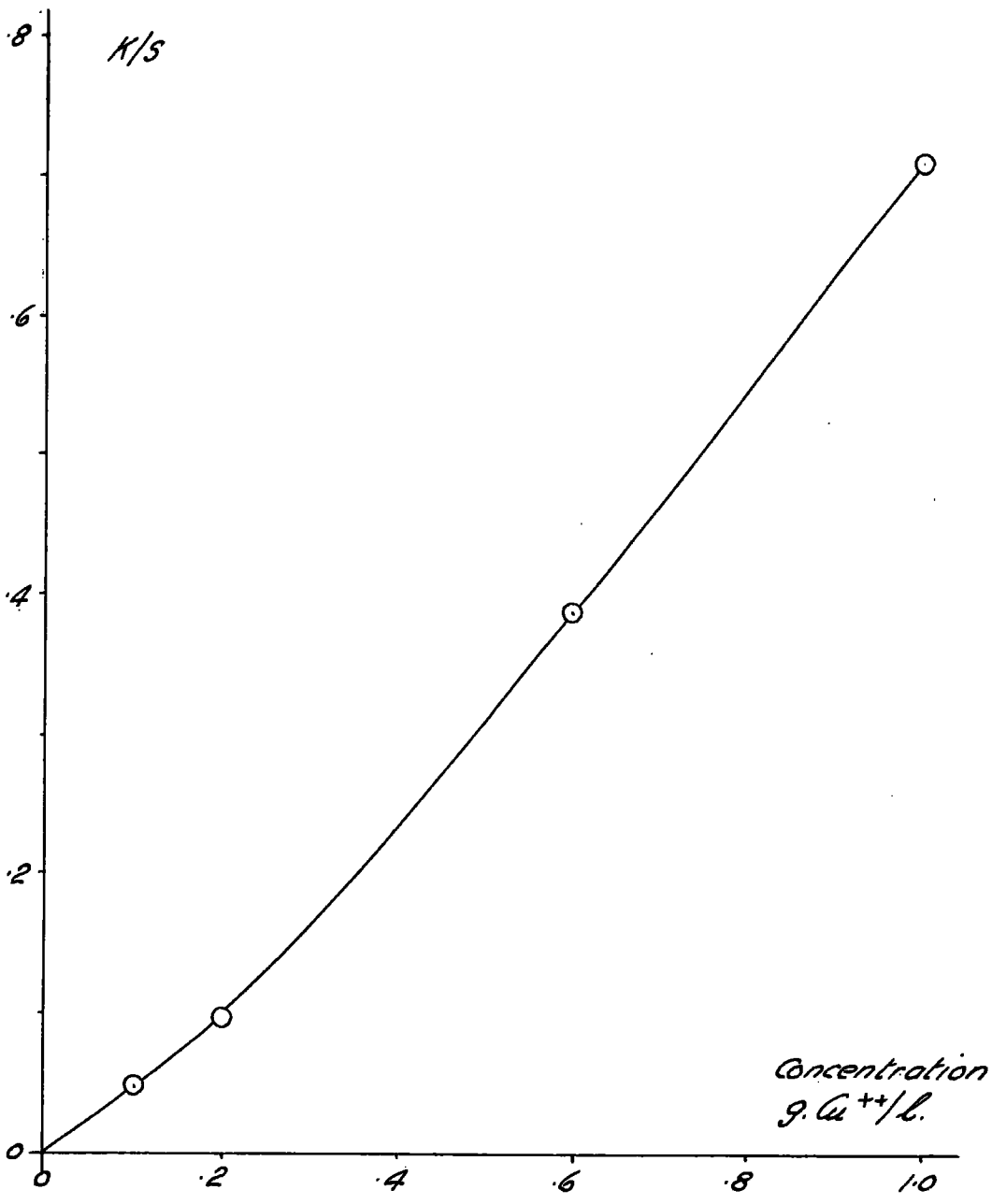

Fig. 30. Calibration curve, reflection method – 2nd. Set.

Table 37

2nd Set

One 'A' sheet and one 'B' sheet run simultaneously [See Table 35]

Temperature of elution 23°C. Time of elution 24 hours.
Volume of spot 2·64μl.

No.of spot	% Reflectance at centre of spot	K/S	Concentration g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l.	
A1	32·3	·709	1.0		
A2	37·5	·521	0·8	0·77	
A3	73·8	·047	0·1		
A4	47·2	·295	0·5	0·49	
A5	42·5	·389	0·6		
A6	58·8	·144	0·25	0·27	
A7	64·3	·099	0·2		
B1	44·2	·352	0·5	·56	*
B2	44·0	·356	0·5	·56	
B3	44·0	·356	0·5	·56	
B4	43·0	·378	0·5	·59	
B5	42·9	·380	0·5	·59	
B6	43·0	·378	0·5	·59	
B7	42·4	·391	0·5	·60	

* Read from curve of 'A' Sheet

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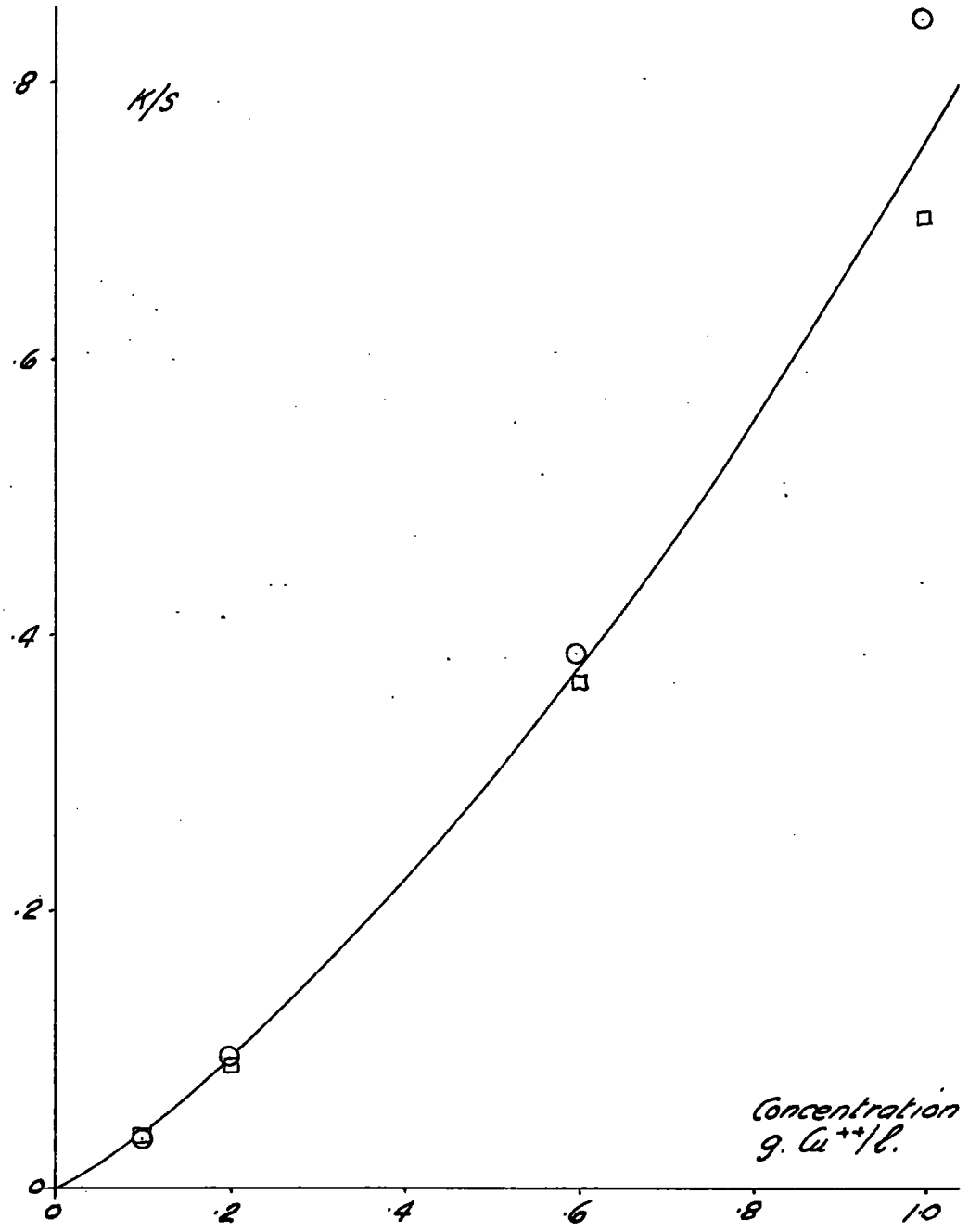

Fig. 31. Calibration curve, reflection method – 3rd. set.

Table 38

3rd Set

Two 'A' sheets run simultaneously [See Table 35]
Temperature of elution 20°C. Time of elution 24 hours.
Volume of spot 2·64 µl.

No.of spot	% Reflectance at centre of spot	K/S	Concentration g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l.
A1	29·4	·848	1·0	
A2	35·3	·593	0·8	·83
A3	76·3	·037	0·1	
A4	46·7	·304	0·5	·52
A5	42·5	·389	0·6	
A6	62·0	·116	0·25	·25
A7	65·1	·094	0·2	
$A^1$1	32·5	0·701	1·0	
$A^1$2	36·1	0·565	0·8	·80
$A^1$3	76·0	0·038	0·1	
$A^1$4	46·0	0·317	0·5	·53
$A^1$5	43·6	0·365	0·6	
$A^1$6	62·5	0·112	0·25	·25
$A^1$7	66·4	0·085	0·2	

(d) Discussion of results of reflection method

The results of the above work are summarised in Tables 39 and 40.

Table 39

Summary of results by reflection method ('A' sheets)

Set	Concentration g.Cu^{++}/l.	Measured concentration g.Cu^{++}/l.	Relative error %	Mean standard relative error %
	0·8	0·75	-6·2	
1A	0·5	0·48	-4·0	$\pm$6·1
	0·25	0·27	+8·0	
	0·8	0·77	-3·8	
2A	0·5	0·49	-2·0	$\pm$3·4
	0·25	0·27	+4·0	
	0·8	0·83	+3·8	
3A	0·5	0·52	+4·0	$\pm$3·2
	0·25	0·25	0	
	0·8	0·80	0	
3A[1]	0·5	0·53	+6·0	$\pm$3·5
	0·25	0·25	0	

Table 40

Summary of results by reflection method ('B' sheets)

Set	Measured concentration g.Cu^{++}/l.	Mean measured concentration g.Cu^{++}/l.	% Relative error from mean	% Mean standard relative error
	0·43		-14	
	0·48		-4	
	0·47		-6	
1B	0·48	0·50	-4	±8·8
	0·57		+14	
	0·54		+ 8	
	0·52		+ 4	
	0·56		3·6	
	0·56		3·6	
2B	0·56	0·58	3·6	±3·0
	0·59		1·7	
	0·59		1·7	
	0·59		1·7	
	0·60		3·6	

The mean standard relative errors of 3 'unknowns' run on the same sheet of paper were 3·4 to 6·1% for single sheets and 3·2 to 3·5% when two sheets were used.

The mean standard relative error for 6 copper spots of the same concentration, separated from widely varying concentrations of nickel and other metals was 3·0 to 8·8%. This indicates that the measured concentration of copper is independent of the presence of the other metals.

D. DISCUSSION OF RESULTS

Preliminary work on non-chromatographed spots showed the importance of developing the spots in ammonia vapour followed by immersion in rubeanic acid, rather than using rubeanic acid containing ammonia. The lack of uniformity of white paper was shown to be much more apparent by reflected than by transmitted light. This suggested that small scanning slit areas should not be used in transmission work. It was found that, even when slits as large as 15 sq. mm. were used in the estimation of non-chromatographed spots, results based on reflection were more precise than those based on transmission. Rendering the paper transparent presented certain advantages both in reflection and transmission work, but was rejected for routine use, owing to the practical difficulties and the increased labour.

Chromatographed spots were scanned by transmission, and it was shown that the minimum transmittance method was as accurate as the method based on the area under the scanning curve. A minimum reflectance method was also investigated which proved to be particularly convenient and equally accurate. The readings were taken in the diffuse reflectance attachment very quickly. This method is recommended for the quantitative estimation of 0·25 to 2·5 μg of copper to an accuracy of $\pm$ 5% in the presence of relatively large quantities of nickel, mercuric mercury, cadmium, and bismuth.

An account of the complete procedure required for the estimation of copper by the reflection method is given below.

Recommended method for estimation of 0·25 to 2·5 μg.Cu^{++} in the presence of relatively large quantities of nickel, mercuric mercury, cadmium, and bismuth.

Prepare standards of concentration 0·1, 0·2, 0·6, 1·0 g. Cu^{++}/l . in 3 N HCl using analytical reagent copper sulphate. Dissolve the substance to be analysed in 3 N HCl so that its copper concentration is between 0·1 g./l. and 1 g./l .

Place the four standards, three samples of the unknown solution, and one blank of 3 N HCl on the starting line of a rectangular sheet of paper 27 cm. wide and 33 cm. long using a micro-pipette of approximately 2·5 μl. capacity. Two similar sheets may be prepared if desired. Allow the sheets to dry and place them in a chromatography tank lined with paper, so that the chromatograms hang from a trough placed near the top of the tank. Add 250 ml. of the organic layer of the solvent, prepared by shaking equal quantities of butanol and 3 N HCl, to the tank, wetting the paper which lines the tank. Close the lid and allow the air in the tank to become saturated with the solvent vapour for 24 hours. Then add 40 ml. of the solvent to the trough through a hole in the lid and elute for 24 hours at a constant temperature of approximately 23°C. Remove the paper from the tank after elution and allow to dry for 2 hours before developing.

Develop the chromatogram by holding in ammonia vapour for half a minute, immersing in rubeanic acid for one minute, holding in ammonia vapour for a further half minute and finally washing in alcohol for half a minute. Allow to dry for at least 5 minutes before measuring.

Cut out each of the spots and measure the reflectance at their centres relative to the blank spot, which is placed

in the standard holder. Plot a graph of K/S (obtained from the reflectance readings) versus concentration using values based on the standards and find the concentration of the unknowns by interpolation. [See Table 38 and Fig. 31]

E. ABSTRACT

A literature survey showed that metals may be estimated both by column chromatography and by many methods employing paper chromatography, including removal of the metal from the paper, direct comparison, measurement of spot size, radioactivity methods, and particularly by photometric estimation directly on paper. Photometric estimations on paper present many practical advantages, but require careful standardisation of conditions. Factors affecting the production of reproducible chromatograms and the principles of photometric estimation on paper are discussed.

The object of the experimental work was to study spectrophotometric methods of estimation directly on paper. A method of ensuring complete and reproducible development of copper and nickel by rubeanic acid is described. The lack of uniformity of paper by transmitted light is shown to be much more apparent than by reflected light and this suggests that very small scanning areas should not be used in transmission work. It is shown that, even when slits as large as 15 sq. mm. are used in the estimation of non-chromatographed spots, results based on reflection are more precise than those based on transmission. Rendering the paper transparent appeared to present some advantages both in reflection and in transmission work, but measurements on dry paper were found to be more suitable for routine use. Chromatograms run on narrow strips were scanned by transmission and estimated both by a minimum transmittance method and by a method involving integration of the total quantity of substance in the spot. Chromatograms run on broad rectangular sheets were estimated by a minimum reflectance method. This method was used in preference to the transmission method of analysis.

The recommended method of estimating 0·25 to 2·5 μg of copper to an accuracy ± 5% in the presence of relatively large quantities of nickel, mercuric mercury, cadmium, and bismuth, involves separation by descending chromatography using butanol 3 N HCl, development in rubeanic acid, cutting out the spots, and measuring the reflectance at their centres at 660 mμ.

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APPENDIX I

Glossary of terms and symbols used

R_f = $\frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$

Elution, the process by which separation is effected

Development, the process of rendering a colourless substance coloured by a reagent such as rubeanic acid

Volume l. ml., µl.

Weight g. mg., µg.

Concentration g./l. (or mg./ml. or µg./µl.)

Wavelength light mµ (millimicrons)

Transmittance = T

Optical density = $\log \frac{1}{T}$

Opacity = 1/T

Reflectance = R

Ratio of absorbtion coefficient to scattering coefficient K/S

Refractive index µ

APPENDIX II

Table* showing ratio of absorption coefficient to scattering coefficient (K/S) as a function of percent reflectance ($100R_\infty$).

*Taken, with kind permission of the publishers, from pp 258-262 of D. B. Judd "Color in Science, Business and Industry", Wiley New York, 1952.

TABLE GIVING K/S AS A FUNCTION OF $100R_\infty$

$100R_\infty$	0	·1	·2	·3	·4	·5	·6	·7	·8	·9
5	9·02	8·83	8·64	8·46	8·29	8·12	7·96	7·80	7·65	7·50
6	7·36	7·23	7·10	6·97	6·84	6·72	6·61	6·50	6·39	6·28
7	6·18	6·08	5·98	5·89	5·79	5·70	5·62	5·53	5·45	5·37
8	5·29	5·21	5·14	5·07	4·99	4·92	4·86	4·79	4·73	4·66
9	4·60	4·54	4·48	4·42	4·37	4·31	4·26	4·20	4·15	4·10
10	4·05	4·00	3·95	3·91	3·86	3·81	3·77	3·73	3·68	3·64
11	3·60	3·56	3·52	3·48	3·44	3·40	3·37	3·33	3·30	3·26
12	3·23	3·19	3·16	3·13	3·09	3·06	3·03	3·00	2·97	2·94
13	2·91	2·88	2·85	2·83	2·80	2·77	2·74	2·72	2·69	2·67
14	2·64	2·62	2·59	2·57	2·54	2·52	2·50	2·47	2·45	2·43
15	2·41	2·39	2·37	2·34	2·32	2·30	2·28	2·26	2·24	2·22
16	2·20	2·19	2·17	2·15	2·13	2·11	2·10	2·08	2·06	2.04
17	2·03	2·01	1·993	1·977	1·961	1·945	1·929	1·913	1·898	1·883
18	1·868	1·853	1·838	1·824	1·809	1·795	1·781	1·767	1·754	1·740
19	1·727	1·713	1·700	1·687	1·674	1·662	1·649	1·637	1·624	1·612
20	1·600	1·588	1·576	1·565	1·553	1·541	1·530	1·519	1·508	1·497
21	1·486	1·475	1·464	1·454	1·443	1·433	1·423	1·413	1·403	1·393
22	1·383	1·373	1·363	1·354	1·344	1·335	1·325	1·316	1·307	1·298
23	1·289	1·280	1·271	1·262	1·254	1·245	1·237	1·228	1·220	1·212
24	1·203	1·195	1·187	1·179	1·171	1·163	1·155	1·148	1·140	1·132
25	1·125	1·117	1·110	1·103	1·095	1·088	1·081	1·074	1·067	1·060
26	1·053	1·046	1·039	1·033	1·026	1·019	1·013	1·006	1·000	0·993
27	0·987	·980	·974	·968	·962	·956	·950	·944	·938	·932
28	0·926	·920	·914	·908	·903	·897	·891	·886	·880	·875
29	0·869	·864	·858	·853	·848	·842	·837	·832	·827	·822
30	0·817	·812	·807	·802	·797	·792	·787	·782	·777	·773
31	0·768	·763	·759	·754	·749	·745	·740	·736	·731	·727
32	0·722	·718	·714	·709	·705	·701	·697	·693	·688	·684
33	0·680	·676	·672	·668	·664	·660	·656	·652	·548	·644
34	0·641	·637	·633	·629	·625	·622	·618	·614	·611	·607
35	0·604	·600	·596	·593	·589	·586	·582	·579	·576	·572
36	0·569	·566	·562	·559	·556	·552	·549	·546	·543	·540
37	0·536	·533	·530	·527	·524	·521	·518	·515	·512	·509
38	0·506	·503	·500	·497	·494	·491	·488	·485	·483	·480
39	0·477	·474	·472	·469	·466	·463	·461	·458	·455	·453
40	0·450	·447	·445	·442	·440	·437	·435	·432	·429	·427
41	0·425	·422	·420	·417	·415	·412	·410	·408	·405	·403
42	0·400	·398	·396	·394	·391	·389	·387	·384	·382	·380
43	0·378	·376	·373	·371	·369	·367	·365	·363	·361	·358
44	0·356	·354	·352	·350	·348	·346	·344	·342	·340	·338
45	0·336	·334	·332	·330	·328	·326	·324	·323	·321	·319
46	0·317	·315	·313	·311	·310	·308	·306	·304	·302	·301
47	0·299	·297	·295	·294	·292	·290	·288	·287	·285	·283
48	0·282	·280	·278	·277	·275	·273	·272	·270	·269	·267
49	0·265	·264	·262	·261	·259	·258	·256	·255	·253	·251

TABLE GIVING K/S AS A FUNCTION OF $100R_\infty$

$100R_\infty$	·0	·1	·2	·3	·4	·5	·6	·7	·8	·9
50	0·250	·249	·247	·246	·244	·243	·241	·240	·238	·237
51	0·235	·234	·233	·231	·230	·228	·227	·226	·224	·223
52	0·222	·220	·219	·218	·216	·215	·214	·212	·211	·210
53	0·208	·207	·206	·205	·203	·202	·201	·200	·198	·197
54	0·196	·195	·194	·192	·191	·190	·189	·188	·186	·185
55	0·184	·183	·182	·181	·180	·178	·177	·176	·175	·174
56	0·173	·172	·171	·170	·169	·167	·166	·165	·164	·163
57	0·162	·161	·160	·159	·158	·157	·156	·155	·154	·153
58	0·152	·151	·150	·149	·148	·147	·146	·145	·144	·143
59	0·142	·142	·141	·140	·139	·138	·137	·136	·135	·134
60	0·133	·132	·132	·131	·130	·129	·128	·127	·126	·126
61	0·125	·124	·123	·122	·121	·121	·120	·119	·118	·117
62	0·116	·116	·115	·114	·113	·112	·112	·111	·110	·109
63	0·109	·108	·107	·106	·106	·105	·104	·103	·103	·102
64	0·101	·101	·100	·099	·098	·098	·097	·096	·096	·095
65	0·094	·094	·093	·092	·092	·091	·090	·090	·089	·088
66	0·088	·087	·086	·086	·085	·084	·084	·083	·083	·082
67	0·081	·081	·080	·079	·079	·078	·078	·077	·076	·076
68	0·075	·075	·074	·074	·073	·072	·072	·071	·071	·070
69	0·070	·069	·069	·068	·067	·067	·066	·066	·065	·065
70	0·064	·064	·063	·063	·062	·062	·061	·061	·060	·060
71	0·059	·059	·058	·058	·057	·057	·056	·056	·055	·055
72	0·054	·054	·054	·053	·053	·052	·052	·051	·051	·050
73	0·050	·049	·049	·049	·048	·048	·047	·047	·047	·046
74	0·046	·045	·045	·044	·044	·044	·043	·043	·042	·042
75	0·042	·041	·041	·041	·040	·040	·039	·039	·039	·038
76	0·038	·038	·037	·037	·036	·036	·036	·035	·035	·035
77	0·034	·034	·034	·033	·033	·033	·032	·032	·032	·031
78	0·031	·031	·030	·030	·030	·029	·029	·029	·029	·028
79	0·028	·028	·027	·027	·027	·026	·026	·026	·026	·025
80	0·025	·025	·024	·024	·024	·024	·023	·023	·023	·023
81	0·022	·022	·022	·022	·021	·021	·021	·020	·020	·020
82	0·020	·020	·019	·019	·019	·019	·018	·018	·018	·018
83	0·017	·017	·017	·017	·017	·016	·016	·016	·016	·015
84	0·015	·015	·015	·015	·014	·014	·014	·014	·014	·013
85	0·013	·013	·013	·013	·012	·012	·012	·012	·012	·012
86	0·011	·011	·011	·011	·011	·011	·010	·010	·010	·010
87	0·010	·009	·009	·009	·009	·009	·009	·009	·008	·008
88	0·008	·008	·008	·008	·008	·007	·007	·007	·007	·007
89	0·007	·007	·007	·006	·006	·006	·006	·006	·006	·006
90	0·006	·005	·005	·005	·005	·005	·005	·005	·005	·005
91	0·004	·004	·004	·004	·004	·004	·004	·004	·004	·004
92	0·003	·003	·003	·003	·003	·003	·003	·003	·003	·003
93	0·003	·003	·002	·002	·002	·002	·002	·002	·002	·002
94	0·002	·002	·002	·002	·002	·002	·002	·001	·001	·001